AD_____

Award Number: W81XWH-06-1-0239

TITLE: A BCR-ABL Kinase Activity-Independent Signaling Pathway in Chronic Myelogenous Leukemia

PRINCIPAL INVESTIGATOR: Shaoguang Li, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Jackson Laboratory
Bar Harbor, Maine 04609-1500

REPORT DATE: February 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-02-2008	Final	1 Feb 2006 – 31 Jan 2008

4. TITLE AND SUBTITLE

A BCR-ABL Kinase Activity-Independent Signaling Pathway in Chronic Myelogenous Leukemia

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0239

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Shaoguang Li, M.D., Ph.D.

Email: shaoguang.li@jax.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Jackson Laboratory
Bar Harbor, Maine 04609-1500

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The BCR-ABL tyrosine kinase inhibitor imatinib mesylate (Gleevec) is the preferred treatment for human chronic myeloid leukemia (CML), but does not cure mice with BCR-ABL-induced acute lymphoblastic leukemia (ALL), similar to CML lymphoid blast crisis. The inability of imatinib to cure CML in mice leads us to hypothesize that a BCR-ABL kinase activity-independent pathway also plays a critical role in the development of this disease. We identified Src kinases as key molecules in this BCR-ABL kinase activity-independent pathway, and they are essential for leukemic cells to survive imatinib treatment and for CML transition to lymphoid blast crisis. Inhibition of both SRC and BCR-ABL kinase activities affords complete B-ALL remission. However, leukemic stem cell pathways must be targeted for curative therapy of Ph+ leukemia. We have identified CML stem cells in mice, and found that these cells are insensitive to imatinib therapy. Our study suggests that Src kinases may be effective in inhibiting leukemic stem cells, and combination therapy using a BCR-ABL/Src inhibitor and an anti-stem cells agent would be beneficial to CML patients. Our work will provide a new therapeutic strategy for CML.

15. SUBJECT TERMS
CML, BCR-ABL ONCOGENE, PH+ LEUKEMIA, MOLECULAR TARGETS, SIGNALING PATHWAY, MOUSE MODEL

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	35	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

Human chronic myelogenous leukemia (CML) is induced by the BCR-ABL oncogene. CML often initiates in a chronic phase and eventually progresses to an advanced terminal blastic phase, in which either acute myeloid or acute lymphoid leukemia (AML or ALL) develops. Thus, a successful treatment of CML requires dealing with diseases in both chronic and advanced or blastic phases. BCR-ABL is an oncogenic kinase. It is generally believed that shutting down the kinase activity of BCR-ABL will completely inhibit its functions, leading to inactivation of its downstream signaling pathways, consequently stopping cellular transformation by BCR-ABL. The BCR-ABL tyrosine kinase inhibitor imatinib mesylate (STI571 or Gleevec) is the preferred treatment for chronic phase CML patients [1]. However, imatinib was unable to abrogate BCR-ABL-expressing leukemic cells [2], and induced cellular and clinical drug resistance [3-9]. Moreover, imatinib is much less effective in treating CML blastic phase patients [10,11], and primitive leukemia cells are insensitive to imatinib treatment [12]. We and others have shown that imatinib prolongs survival of mice with BCR-ABL-induced CML [13,14], but does not cure the disease [13]. We have also shown that imatinib does not cure mice with BCR-ABL-induced ALL, similar to CML lymphoid blast crisis [13]. Because imatinib is a strong inhibitor against BCR-ABL kinase activity, the inability of imatinib to cure CML and ALL in mice [13] leads us to hypothesize that a BCR-ABL kinase activity-independent pathway also plays a critical role in the development of CML. Our preliminary data suggested that in this application, we would test our hypotheses using mouse models of human CML [15-17] and ALL [15,18]. Specifically, we would study the role of this kinase-independent pathway in the development of chronic phase CML and advanced phase CML represented by B-ALL, and test if targeting this pathway is therapeutically effective.

Body

With the support from this grant, we were able to complete the projects outlined in the Statement of Work. Below, I describe in detail what we have accomplished during two years with the support from the Department of Defense (DOD). The results for all completed experiments have been either described here by words or published (Hu Y, Swerdlow S, Duffy TM, Weinmann R, Lee FY, Li S. Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph^+ leukemia. **Proc Natl Acad Sci USA** 103(45):16870-16875, 2006), and corresponding numbers of figures in this paper are used below.

Task 1. To determine the effect of Lyn, Hck, and Fgr in survival and self-renewal of BCR-ABL-expressing hematopoietic stem cells (HSCs):

a. Develop a FACS analysis to identify BCR-ABL-expressing HSCs.

By FACS analysis, we had successfully identified BCR-ABL-expressing hematopoietic stem cells (HSCs) as CML stem cells in mice. These stem cells are $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (Fig. 6B).

b. Determine the effect of Lyn, Hck, and Fgr in survival and self-renewal of BCR-ABL-expressing HSCs using Src knockout mice.

Bone marrow cells from $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ or wild type C57BL/6 (B6) mice were transduced with BCR-ABL retrovirus, followed by transplantation into B6 recipient mice to induce CML. To study the role of these Src kinases in survival regulation of CML stem cells (BCR-ABL-expressing HSCs), bone marrow cells were isolated from these CML mice, and compared the percentages of $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ and wild type BCR-ABL-expressing HSCs in side population (SP) [19]. We found that the percentage of BCR-ABL-expressing HSCs in SP in recipients of BCR-ABL-transduced $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ bone marrow cells was significantly less than that in recipients of BCR-ABL-transduced wild type bone marrow cells, suggesting that lack of Lyn, Hck, and Fgr causes a reduction of CML stem cells. This conclusion needs to be further supported by more detailed *in vivo* FACS analysis in the future.

c. Demonstrate the role of Src kinases in survival and self-renewal of BCR-ABL-expressing HSCs using the dual Src/BCR-ABL kinase inhibitor BMS-354825.

This experiment is to provide further support for the genetic determination of the effect of Lyn, Hck, and Fgr in survival and self-renewal of BCR-ABL-expressing HSCs using Src knockout mice (described in Task 1b above). We treated CML mice with BMS-354825 (Dasatinib), and analyzed BCR-ABL-expressing HSCs in SP compare to those of placebo-treated CML mice. BMS-354825 treatment slightly reduced the percentage of BCR-ABL-expressing HSCs in SP (Fig. 5C), suggesting that inhibition of Src kinases may have an inhibitor effect against CML stem cells.

Task 2. To test whether lack of Lyn, Hck, and Fgr prevents transition of CML chronic phase to lymphoid blast crisis:

a. Test whether Src kinases play a role in CML transition to lymphoid blast crisis using Src knockout mice in a serial transplantation assay.

We tested whether Src kinases play a role in CML transition to lymphoid blast crisis using a serial transplantation assay [16]. Mice were transplanted with BCR-ABL-transduced bone marrow (BM) cells from either wild type or $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ mice to induce CML, and BM cells from the CML mice were transferred into lethally irradiated syngeneic recipient mice. Mice receiving wild type CML BM cells developed ALL diagnosed by FACS using $GFP^+CD43^+B220^+CD19^+$ as markers, whereas mice receiving $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ CML BM cells did not develop this disease (Fig. 3E). These results demonstrate that lack of Lyn, Hck, and Fgr prevents transition of CML chronic phase to lymphoid blast crisis.

b. Test whether Src kinases play a role in CML transition to lymphoid blast crisis using BMS-354825 in a serial transplantation assay.

This experiment is to further demonstrate our finding through genetic approach (described in the Task 2a above), which shows a role of Src kinases in CML transition to lymphoid blast crisis. Due to the shortage of BMS-354825 acquired from the company, we were unable to perform this experiment. Instead, we used CML stem cells identified under this grant support to study inhibition of CML stem cells by inhibition of heat shock protein with an inhibitor. This study has been published recently in the journal of Blood (see the attached paper in Appendices), and the DOD support has been mentioned in this paper.

Task 3. To determine whether activation of Src kinases by BCR-ABL provides a mechanism for insensitivity of ALL in CML blastic phase to imatinib treatment:

a. Demonstrate the role of Src kinases in causing the insensitivity of ALL to imatinib using wild type BCR-ABL and BMS-354825.

We treated ALL mice with BMS-354825, which inhibits both BCR-ABL and Src kinase activity, or with imatinib, which only inhibits BCR-ABL kinase activity. We found that BMS-354825 more markedly prolongs survival of the mice than imatinib did (Fig. 3A). The therapeutic effects of these two drugs correlated with reduced levels of GFP^+ leukemic cells in peripheral blood of the treated mice (Fig. 3B). To demonstrate that the weaker therapeutic effect of imatinib is not due to a failure of imatinib to inhibit BCR-ABL kinase activity *in vivo*, we examined if imatinib significantly inhibits BCR-ABL phosphorylation in pre-B leukemic cells from peripheral blood of the treated mice. We found that imatinib and BMS-354825 similarly inhibited BCR-ABL phosphorylation in mice (Fig. 3C). These results demonstrate that Src kinases are responsible for causing the insensitivity of ALL to imatinib.

b. Demonstrate the role of Src kinases in causing the insensitivity of ALL to imatinib using the BCR-ABL-T315I mutant and BMS-354825.

We treated mice with BCR-ABL-T315I-induced ALL with BMS354825 or imatinib to compare survival of the two treatment groups of mice, allowing only investigating the effect of Src inhibition on ALL, because BCR-ABL-T315I mutant is no longer sensitive to inhibition of its kinases activity by

imatinib and BMS-354825, but its activation of Src kinases is not affected and can be inhibited by BMS-435825. We found that inhibition of Src kinases by BMS-435825 significantly prolonged survival of B-ALL mice, but imatinib did not (Fig. 2D), demonstrating that the ineffectiveness of imatinib in treating ALL mice was due to the Src kinase activation that was not inhibited by imatinib.

Key Research Accomplishments

1. Establishment of a critical role of Src kinases in the development of advanced CML.
2. Identification of CML stem cells in mice.
3. Elucidation of the role of Src kinases in regulation of CML stem cells.
4. A combination therapeutic strategy for treating CML: anti-CML stem cells in combination with anti-proliferation of more differentiated leukemic cells (see attached published paper).

Reportable Outcomes

The results have been recently published in the journals of (1) PNAS (Hu Y, Swerdlow S, Duffy TM, Weinmann R, Lee FY, **Li S**. Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph^+ leukemia. **Proc Natl Acad Sci USA** 103(45):16870-16875, 2006); (2) **Blood** (Peng C, Brain J, Hu Y, Goodrich A, Kong L, Grayzel D, Pak R, Read M, **Li S**. Inhibition of heat shock protein 90 prolongs survival of mice with BCR-ABL-T315I-induced leukemia and suppresses leukemic stem cells. **Blood** 110(2):678-685, 2007); and (3) J. Cell. Mol. Med. (**Li S,** Li D**.** Stem cell and kinase activity-independent pathway in resistance of leukemia to BCR-ABL kinase inhibitors. **J. Cell. Mol. Med.** 11(6):1251-1262, 2007).

Conclusions

Src kinases are essential for leukemic cells to survive imatinib treatment and for CML transition to lymphoid blast crisis. Src kinases are valuable therapeutic targets, and inhibition of both Src and BCR-ABL kinase activities by BMS-354825 is critical to ALL treatment. Src kinases may also play a critical role in regulation of CML stem cells.

References

1. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001;344:1031-1037.
2. Marley SB, Deininger MW, Davidson RJ, Goldman JM, Gordon MY. The tyrosine kinase inhibitor STI571, like interferon-alpha, preferentially reduces the capacity for amplification of granulocyte-macrophage progenitors from patients with chronic myeloid leukemia. Exp Hematol. 2000;28:551-557.
3. Gorre ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science. 2001;293:876-880.
4. Weisberg E, Griffin JD. Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines. Blood. 2000;95:3498-3505.
5. le Coutre P, Tassi E, Varella-Garcia M, et al. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. Blood. 2000;95:1758-1766.
6. Mahon FX, Deininger MW, Schultheis B, et al. Selection and characterization of *BCR-ABL* positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. Blood. 2000;96:1070-1079.
7. Shah NP, Nicoll JM, Nagar B, et al. Multiple *BCR-ABL* kinase domain mutants confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crise chronic myeloid leukemia. Cancer Cell. 2002;2:117-125.

8. Branford S, Rudzki Z, Walsh S, et al. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood. 2002;99:3472-3475.
9. von Bubnoff N, Schneller F, Peschel C, Duyster J. BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study. Lancet. 2002;359:487-491.
10. Druker BJ, Sawyers CL, Kantarjian H, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med. 2001;344:1038-1042.
11. Talpaz M, Sawyers CL, Kantarjain H, et al. Activity of an ABL specific tyrosine kinase inhibitor in patients with BCR/ABL positive acute leukemias, including chronic myelogenous leukemia in blast crisis. Oncologist. 2000;5:282-283 (Abstr.).
12. Holtz MS, Slovak ML, Zhang F, Sawyers CL, Forman SJ, Bhatia R. Imatinib mesylate (ST1571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation. Blood. 2002;99:3792-3800.
13. Hu Y, Liu Y, Pelletier S, et al. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. Nat Genet. 2004;36:453-461.
14. Wolff NC, Ilaria RL, Jr. Establishment of a murine model for therapy-treated chronic myelogenous leukemia using the tyrosine kinase inhibitor STI571. Blood. 2001;98:2808-2816.
15. Li S, Ilaria RL, Jr., Million RP, Daley GQ, Van Etten RA. The P190, P210, and p230 forms of the *BCR/ABL* oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. J Exp Med. 1999;189:1399-1412.
16. Pear WS, Miller JP, Xu L, et al. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood. 1998;92:3780-3792.
17. Zhang X, Ren R. Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. Blood. 1998;92:3829-3840.
18. Roumiantsev S, de Aos IE, Varticovski L, Ilaria RL, Van Etten RA. The src homology 2 domain of Bcr/Abl is required for efficient induction of chronic myeloid leukemia-like disease in mice but not for lymphoid leukemogenesis or activation of phosphatidylinositol 3-kinase. Blood. 2001;97:4-13.
19. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996;183:1797-1806.

Table of Appendices

PNAS
Proceedings of the National Academy of Sciences of the United States of America www.pnas.org

Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph + leukemia in mice

Yiguo Hu, Sarah Swerdlow, Theodore M. Duffy, Roberto Weinmann, Francis Y. Lee, and Shaoguang Li

PNAS 2006;103;16870-16875; originally published online Oct 31, 2006;
doi:10.1073/pnas.0606509103

Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph$^+$ leukemia in mice

Yiguo Hu*, Sarah Swerdlow*, Theodore M. Duffy*, Roberto Weinmann†, Francis Y. Lee†, and Shaoguang Li*‡

*The Jackson Laboratory, Bar Harbor, ME 04609; and †Bristol-Myers Squibb Oncology, Princeton, NJ 08543

Edited by Charles J. Sherr, St. Jude Children's Research Hospital, Memphis, TN, and approved September 25, 2006 (received for review August 9, 2006)

It is generally believed that shutting down the kinase activity of BCR-ABL by imatinib will completely inhibit its functions, leading to inactivation of its downstream signaling pathways and cure of the disease. Imatinib is highly effective at treating human Philadelphia chromosome-positive (Ph$^+$) chronic myeloid leukemia (CML) in chronic phase but not Ph$^+$ B cell acute lymphoblastic leukemia (B-ALL) and CML blast crisis. We find that SRC kinases activated by BCR-ABL remain fully active in imatinib-treated mouse leukemic cells, suggesting that imatinib does not inactivate all BCR-ABL-activated signaling pathways. This SRC pathway is essential for leukemic cells to survive imatinib treatment and for CML transition to lymphoid blast crisis. Inhibition of both SRC and BCR-ABL kinase activities by dasatinib affords complete B-ALL remission. However, curing B-ALL and CML mice requires killing leukemic stem cells insensitive to both imatinib and dasatinib. Besides BCR-ABL and SRC kinases, stem cell pathways must be targeted for curative therapy of Ph$^+$ leukemia.

dasatinib | imatinib | SRC kinases

The human Philadelphia chromosome, Ph, arises from a translocation between chromosomes 9 and 22 and results in formation of the chimeric and constitutively activated BCR-ABL tyrosine kinase. Philadelphia chromosome-positive (Ph$^+$) leukemias induced by the BCR-ABL oncogene include chronic myeloid leukemia (CML) and B cell acute lymphoblastic leukemia (B-ALL). CML often initiates in a chronic phase and eventually progresses to a terminal blastic phase in which either acute myeloid or acute B-lymphoid leukemia develops. Some Ph$^+$ leukemia patients, however, have B-ALL as their first clinical appearance. It is generally believed that shutting down the kinase activity of BCR-ABL will completely inhibit its functions, leading to inactivation of its downstream signaling pathways. Therefore, current therapeutic efforts have focused on targeting BCR-ABL kinase activity by using kinase inhibitors.

The BCR-ABL tyrosine kinase inhibitor imatinib mesylate (also known as Gleevec) is the standard of care for Ph$^+$ leukemia. Imatinib induces a complete hematologic response in chronic-phase CML patients (1). However, imatinib does not completely eliminate BCR-ABL-expressing leukemic cells (2, 3), and patients frequently present with drug resistance (4). Imatinib prolongs survival of mice with BCR-ABL-induced CML (5, 6) but does not cure the disease (5). Recently, three BCR-ABL kinase inhibitors, dasatinib (7), AP23464 (8), and AMN107 (9), have been shown to inhibit almost all imatinib-resistant BCR-ABL mutants; the exception is the T315I mutant, which is present in 15–20% of imatinib-resistant patients. Dasatinib also is a potent inhibitor of SRC family kinases, but the role of the anti-SRC activity of this compound in Ph$^+$ leukemia therapy has not been studied (7). For unknown reasons, imatinib is much less effective in treating CML blastic-phase patients and patients with Ph$^+$ B-ALL (10), which has not been shown to be related to the BCR-ABL kinase domain mutations, the most common type of imatinib resistance. Because imatinib is a strong inhibitor of BCR-ABL kinase activity, the inability of imatinib to cure CML and B-ALL in mice (5) suggests that inactivation of BCR-ABL kinase activity alone is insufficient to control the disease.

We have previously shown that the three SRC-family kinases, LYN, HCK, and FGR, are activated by BCR-ABL in lymphoid leukemic cells and are required for the development of B-ALL (5). Furthermore, cells from patients resistant to imatinib expressed an activated form of LYN (11). We reasoned that inhibition of BCR-ABL kinase activity by imatinib might not inactivate SRC kinases activated by BCR-ABL in lymphoid leukemic cells, and this may explain the relatively poor activity of imatinib against Ph$^+$ B-ALL and lymphoid blast crisis. In this study, we provide evidence that imatinib does not inactivate the SRC signaling pathway activated by BCR-ABL and that this pathway is essential for the development of Ph$^+$ B-ALL. We also show that other targets need to be identified to inhibit imatinib-insensitive leukemic stem cells for Ph$^+$ B-ALL and CML.

Results

SRC Kinases Remain Active After Imatinib Inhibition of BCR-ABL Kinase Activity. We tested the hypothesis that imatinib may not inactivate SRC kinases activated by BCR-ABL using a BCR-ABL-expressing pre-B cell line (ENU) (5). The cells were treated with or without imatinib. Compared with cells bearing the empty vector, Western blot analysis showed that SRC kinases were activated in cells expressing one of two forms of BCR-ABL (P190 and P210, which differ in molecular weight and are expressed predominantly in Ph$^+$ ALL and CML, respectively), and imatinib treatment markedly inhibited BCR-ABL kinase activity but did not result in a decrease in SRC activation (Fig. 1*A*). These results indicate that, although imatinib was very effective in inhibiting BCR-ABL phosphorylation, it was unable to affect BCR-ABL-stimulated phosphorylation of SRC kinases. To demonstrate this finding further, we used the P190 or P210 form of BCR-ABL to transform mouse bone marrow (BM) cells. These cells were then treated with imatinib. Imatinib inhibited BCR-ABL phosphorylation, resulting in decreased phosphorylation of downstream signaling molecule CrkL, but did not affect BCR-ABL-stimulated phosphorylation of SRC kinases (Fig. 1*B*). These observations indicate that, in imatinib-treated BCR-ABL-expressing cells, SRC kinases are still active and may participate in cellular transformation by BCR-ABL.

Author contributions: S.L. designed research; Y.H., S.S., and S.L. performed research; R.W. and F.Y.L. contributed new reagents/analytic tools; Y.H., T.M.D., and S.L. analyzed data; and S.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Freely available online through the PNAS open access option.

Abbreviations: B-ALL, B cell acute lymphoblastic leukemia; BM, bone marrow; CML, chronic myeloid leukemia; HSC, hematopoietic stem cell.

‡To whom correspondence should be addressed. E-mail: shaoguang.li@jax.org.

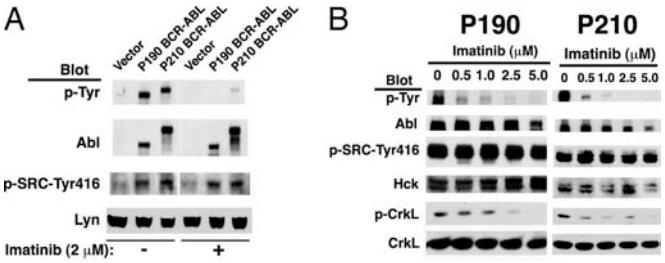

Fig. 1. SRC kinases remain active after inhibition of BCR-ABL kinase activity by imatinib. (*A*) Activation of SRC kinases by BCR-ABL does not require BCR-ABL kinase activity. P190- or P210-expressing ENU cells were cultured in the presence or absence of imatinib for 12 h, and ENU cells bearing empty vector were used as controls. Protein lysates were analyzed by Western blotting with antibodies against phosphotyrosine (*p*-Tyr), ABL, activated SRC kinases (*p*-SRC-Tyr-416) (29), and LYN. (*B*) BCR-ABL-transduced BM cells were cultured under Whitlock–Witte conditions for 5 days. The cells were treated with imatinib at the concentrations indicated for 2 days. Protein lysates were analyzed by Western blotting with the antibodies indicated.

Key Role of SRC Kinases in Malignant Transformation of B-Lymphoid Cells. To investigate the role of SRC kinase activation in transformation of B-lymphoid cells and in the survival and proliferation of leukemic cells, we showed that v-SRC, an active form of SRC kinase, directly transformed B-lymphoid cells *in vitro* (Fig. 2*A*), suggesting that activated SRC alone is sufficient to stimulate aberrant proliferation of hematopoietic precursors. To examine whether inhibition of SRC kinases attenuates transformation of mouse BM cells by BCR-ABL, we used a dual SRC/BCR-ABL inhibitor dasatinib (Fig. 2*B*). We transformed mouse BM cells with the BCR-ABL-T315I mutant that is resistant to inhibition of BCR-ABL kinase activity by both imatinib (4, 12, 13) and dasatinib (7). This allowed us to dissociate the inhibitory effects on the BCR-ABL kinase vs. the effects on SRC kinases. Dasatinib reduced survival (Fig. 2*C*) and induced apoptosis (data not shown) of the leukemic cells, demonstrating that BCR-ABL-activated SRC kinases in imatinib-treated cells play a critical role in BCR-ABL-mediated transformation of B-lymphoid cells.

We further investigated the role of SRC kinase in B-ALL development using a mouse model of B-ALL (14). We treated mice with B-ALL induced by BCR-ABL-T315I with imatinib or dasatinib. Imatinib showed no therapeutic effect, whereas dasatinib significantly prolonged survival of the mice ($P < 0.01$) (Fig. 2*D*). Dasatinib inhibited SRC kinase activity *in vivo* (Fig. 2*E*). These results indicate that SRC kinases play a critical role in B-ALL development. However, targeting SRC kinases alone did not cure the disease (Fig. 2*D*), which may be due to the incomplete inhibition of SRC kinase activity *in vivo* at the dose of dasatinib used (Fig. 2*E*); a higher dose of dasatinib may further improve survival of the mice. To further support the role of SRC kinases in B-ALL development, we compared growth potential of BCR-ABL-transduced wild-type and $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ BM cells. We monitored the levels of pre-B leukemic cells expressing BCR-ABL (represented by GFP expression) over a 4-week time period in peripheral blood of mice receiving BCR-ABL-transduced wild-type or $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ BM cells. Levels of GFP^+B220^+ B-lymphoid leukemic cells were significantly lower in mice receiving BCR-ABL-transduced $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ BM cells than in those receiving BCR-ABL-transduced wild-type BM cells at all time points measured, although there was an initial increase in leukemic cells in mice receiving the transduced $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ BM cells (Fig. 2*F*). Strikingly, in mice receiving the transduced $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ BM cells, leukemic cells almost disappeared 5 weeks following B-ALL induction, whereas in the mice receiving the transduced wild-type BM cells, ≈45% of leukemic cells persisted (Fig. 2*G*).

Inhibition Solely of BCR-ABL Kinase Activity Without SRC Kinase Inhibition Is Insufficient for B-ALL Treatment. Because SRC kinases are still active when BCR-ABL phosphorylation is inhibited by

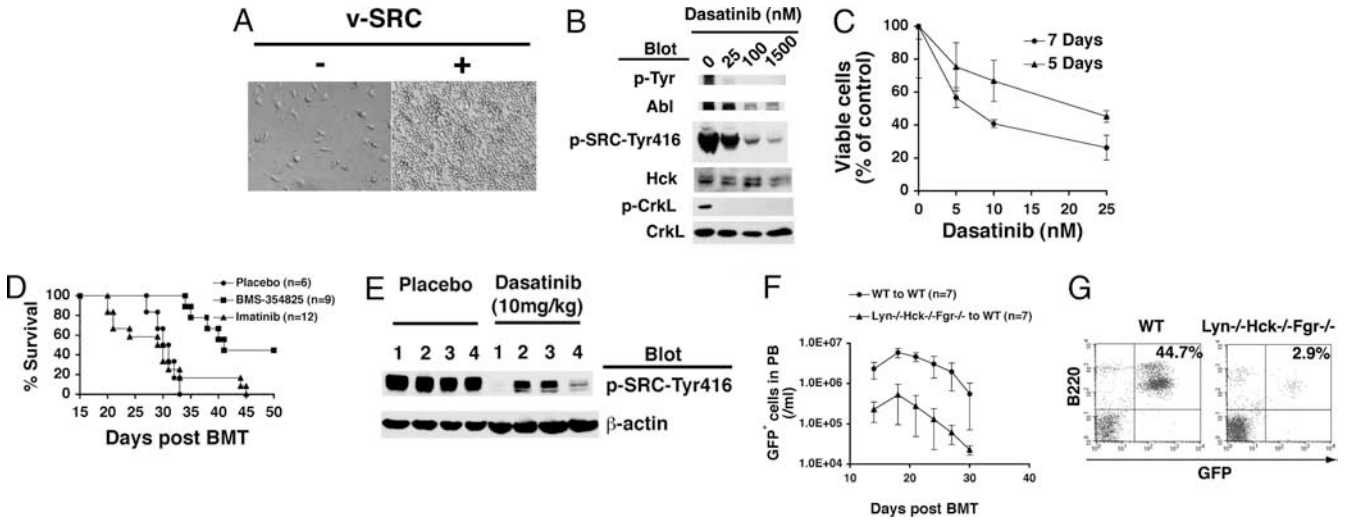

Fig. 2. SRC kinases play a critical role in maintaining survival and promoting proliferation of pre-B leukemic cells. (*A*) BM cells from B6 mice were transduced with the empty vector or v-SRC retrovirus and cultured under Whitlock–Witte conditions for 14 days. (*B*) Dasatinib inhibits activity of both BCR-ABL and SRC kinases. BCR-ABL-transduced BM cells were cultured under Whitlock–Witte conditions for 5 days. Different concentrations of dasatinib were added to the culture for 48 h, and protein lysates were analyzed by Western blotting. (*C*) Inhibition of SRC kinases reduces survival of BCR-ABL-T315I-expressing B-lymphoid cells. BCR-ABL-T315I-transduced BM cells were cultured at 1×10^5 cells per well in 24-well plates, and different concentrations of dasatinib were added to the culture for 5 or 7 days. Viable cells were counted. (*D*) Therapeutic effect of imatinib and dasatinib on BCR-ABL-T315I-induced B-ALL. BMT, BM transplantation. (*E*) *In vivo* inhibition of SRC kinase activity with dasatinib. Mice with BCR-ABL-T315I-induced B-ALL were treated with a placebo or dasatinib for 3 days. After the last dose, leukemic cells from peripheral blood of the mice were analyzed by Western blotting. Each lane represents a mouse from the indicated treatment group. (*F*) BCR-ABL-transduced wild-type or Lyn/Hck/Fgr triple knockout BM cells were transplanted into wild-type recipient mice to induce B-ALL. GFP^+ cell counts (percentage of GFP^+ cells × white blood cell count) were measured at different time points after the induction of leukemia. (*G*) Percentages of GFP^+ B-leukemic cells in peripheral blood were determined by FACS analysis as described in *F*.

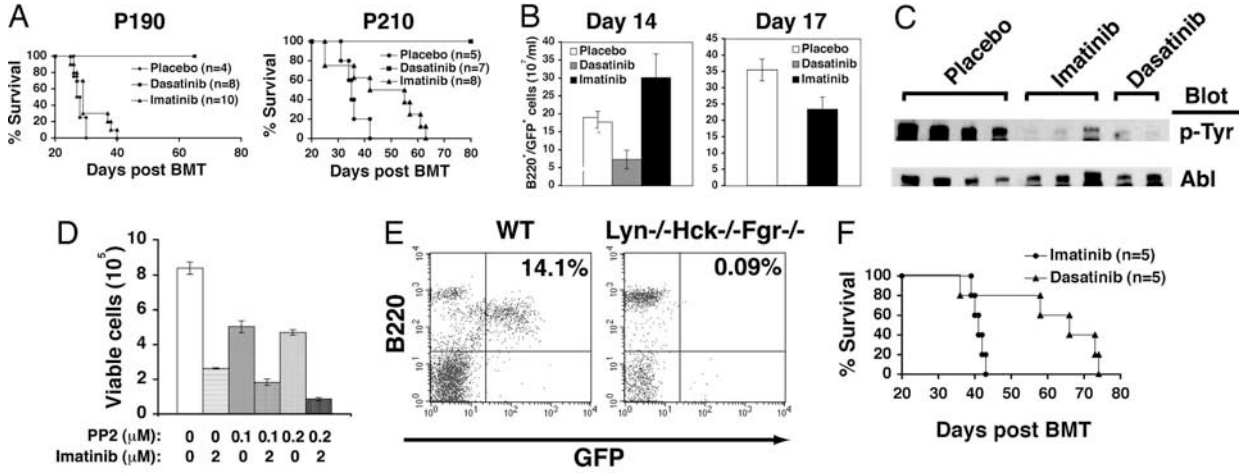

Fig. 3. Simultaneous targeting of kinase activity of both BCR-ABL and SRC kinases results in long-term survival of mice with B-ALL. (*A*) Mice with BCR-ABL-induced B-ALL were treated with a placebo, imatinib, or dasatinib. BMT, BM transplantation. (*B*) Reduction of GFP^+ leukemic cells in peripheral blood of the treated B-ALL mice. (*C*) *In vivo* inhibition of BCR-ABL autophosphorylation by imatinib and dasatinib. B-ALL mice were treated with placebo, imatinib, or dasatinib for 3 days. After the last dose, leukemic cells from the pleural effusion were analyzed by Western blotting. Each lane represents a mouse from the indicated treatment group. (*D*) The SRC-selective kinase inhibitor PP2 alone or with imatinib has an inhibitory effect on proliferation of BCR-ABL-transduced BM cells in Whitlock–Witte culture. The transduced cells were cultured at 1×10^5 per well in 24-well plates for 5 days, and the two drugs were added to the culture for the last 2 days. Viable cells were counted. (*E*) Lack of LYN, HCK, and FGR prevents CML transition to lymphoid blast crisis. Wild-type and Lyn/Hck/Fgr triple knockout BM cells from CML mice were transferred into wild-type recipient mice to assay CML transition to B-ALL by FACS analysis of GFP^+ B-leukemic cells in peripheral blood. (*F*) Dasatinib, but not imatinib, is effective at suppressing p53-deficient leukemic cells in B-ALL mice.

imatinib (Fig. 1), we examined whether inhibition of BCR-ABL kinase activity alone, with SRC kinase still active, is sufficient to control B-ALL. We treated mice with BCR-ABL-induced B-ALL with imatinib (which inhibits only BCR-ABL kinase activity) or with dasatinib (which inhibits both BCR-ABL and SRC kinase activity). Imatinib had a weak therapeutic effect on B-ALL (Fig. 3*A*), suggesting that inhibition solely of BCR-ABL kinase activity is insufficient to control the disease. By contrast, dasatinib maintained long-term survival of the mice with B-ALL induced by P190 or P210 BCR-ABL (Fig. 3*A*), indicating that both BCR-ABL kinase activity and SRC pathway must be targeted for treating this disease. The therapeutic effects of these two drugs on B-ALL correlated with reduced levels of GFP^+ leukemic cells in peripheral blood of the treated B-ALL mice (Fig. 3*B*). The weak therapeutic effect of imatinib (Fig. 3*A*) cannot be attributed to an inability to inhibit BCR-ABL kinase activity *in vivo*, because imatinib significantly inhibited BCR-ABL phosphorylation to a similar extent compared with dasatinib in leukemic cells from pleural effusion of the treated B-ALL mice (Fig. 3*C*). To exclude the possibility that the better therapeutic effect of dasatinib over imatinib (Fig. 3*A*) could be attributed to the potency difference of these drugs on BCR-ABL kinase activity (7) but not to the additional anti-SRC effect of dasatinib (Fig. 2*B*), we treated BCR-ABL-transformed BM cells under Whitlock–Witte conditions with a SRC kinase inhibitor PP2 alone (15) (which did not inhibit BCR-ABL kinase activity at the concentrations used in this study), with imatinib alone, and with both PP2 and imatinib. Either drug alone inhibited proliferation of the cells, but both drugs together had a much stronger inhibitory effect (Fig. 3*D*). These results support the critical role of SRC kinases in B-ALL development.

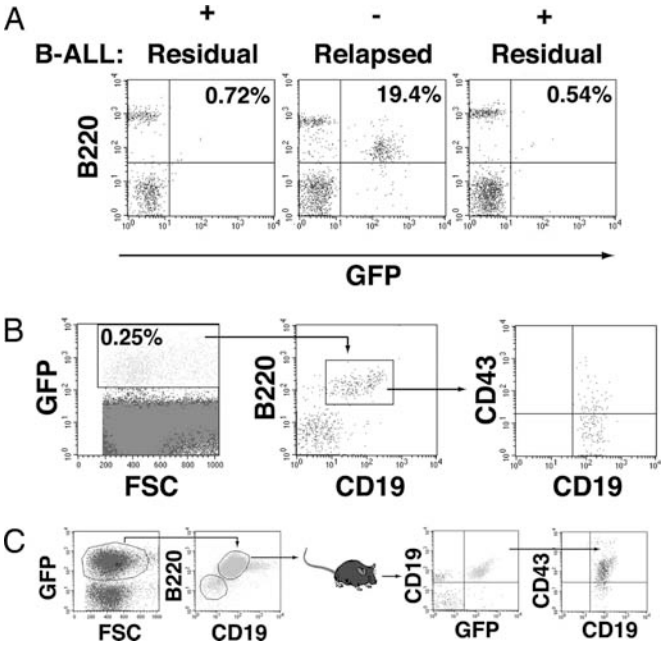

Fig. 4. Dasatinib efficiently kills highly proliferating B-leukemic cells, but not stem cells, in B-ALL mice. (*A*) B-ALL reappeared in most of the mice after dasatinib treatment stopped (−); the relapsed mice remained sensitive to dasatinib therapy (+). (*B*) A low level of GFP^+ pro- or pre-B cells (<1%) persisted in dasatinib-treated mice. (*C*) $B220^+/CD43^+$ pro-B leukemic cells function as leukemic stem cells in B-ALL. The sorted $GFP^+/B220^+/CD19^+$ cells from BM of B-ALL mice transfer B-ALL to secondary recipients after 2 months, and leukemic cells in peripheral blood are $B220^+/CD43^+$ pro-B cells.

Progression to Lymphoid Blast Crisis CML Requires Activation of SRC Kinases. Chronic-phase CML advances to blastic phase. We genetically tested whether SRC kinases play a role in CML transition to lymphoid blast crisis using a serial transplantation assay (16). Mice were transplanted with BCR-ABL-transduced BM cells from either wild-type or $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ mice to induce CML, and BM cells from the CML mice were subsequently transferred into recipient mice. Mice receiving wild-type CML BM cells developed B-ALL, shown by $GFP^+/B220^+$ leukemic cells in peripheral blood, whereas none of the mice receiving $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ CML BM cells developed this disease (Fig. 3*E*). These results indicate that CML transition to

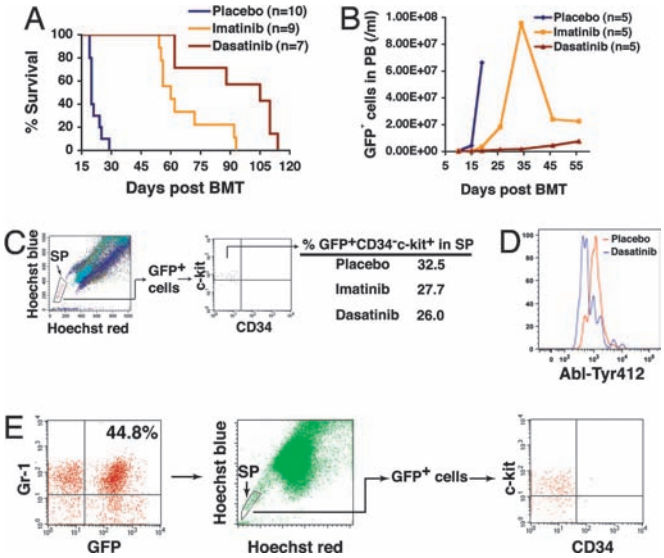

Fig. 5. Imatinib and dasatinib fail to eradicate BCR-ABL-expressing HSCs completely. (*A*) CML mice treated with imatinib and dasatinib. BMT, BM transplantation. (*B*) GFP^+ leukemic cell counts in peripheral blood (PB) of CML mice treated with imatinib and dasatinib. (*C*) Comparison of the percentages of BCR-ABL-expressing HSCs (GFP^+CD34^-c-*kit*$^+Hoe^-$) in side populations (SP) of BM cells from placebo-, imatinib-, and dasatinib-treated CML mice. (*D*) Dasatinib inhibits BCR-ABL kinase activity in CML stem cells. BM cells from CML mice were treated with a placebo or dasatinib (100 nM) in culture for 24 h, and BCR-ABL-expressing HSCs (GFP^+CD34^-c-*kit*$^+Hoe^-$) were identified by FACS. Intracellular levels of BCR-ABL phosphorylation were determined by FACS with anti-Abl-Y412 antibody, which detects the active form of BCR-ABL. (*E*) A representative CML mouse treated with dasatinib for 16 weeks still contains large numbers of BCR-ABL-expressing HSCs.

lymphoid blast crisis requires SRC kinases. CML progression is associated with additional genetic changes, including mutations in the tumor suppressor genes INK4[a], pRB, and p53 (17–19). A recent study showed that Arf gene loss enhances the oncogenicity of imatinib and limits imatinib response to BCR-ABL-induced B-ALL in mice (20). To test whether SRC kinases are effective targets for B-ALL when tumor suppressor gene function is defective, BCR-ABL-transduced BM cells from p53-deficient mice were transplanted into lethally irradiated wild-type recipient mice followed by treatment with imatinib or dasatinib. Dasatinib was more effective than imatinib in suppressing p53-deficient pre-B leukemic cells, but these mice eventually died (Fig. 3*F*). These results suggest that loss of p53 function causes reduction of dasatinib response to BCR-ABL-induced B-ALL, although a significant degree of response remained.

Pro-B Leukemic Cells Are Identified as B-ALL Stem Cells, and Continuous Treatment with Dasatinib May Prevent Them from Developing into B-ALL. We tested whether dasatinib could completely eradicate leukemic cells in B-ALL mice, leading to cures. Although dasatinib remarkably prolonged survival of B-ALL mice (Fig. 3*A*), a small percentage of GFP^+ cells (<1%) remained in the peripheral blood of these mice even after 3 months of treatment (Fig. 4*A*). After treatment was stopped, B-ALL reappeared in most mice (Fig. 4*A*) within 1 month for P190BCR-ABL-induced and within 2 months for P210BCR-ABL-induced B-ALL. The relapsed B-ALL mice were treated again with dasatinib, and the percentage of GFP^+ cells dropped again to <1% (Fig. 4*A*) and remained at this level during continuous drug treatment for 2 months (data not shown). After two rounds of treatment discontinuations, relapses, and retreatment, the mice remained sensitive to the next round of dasatinib therapy (data not shown). Still, a low level (<1%) of GFP^+ cells persisted in the BM of the treated B-ALL mice, and these cells were capable of transferring the same disease to secondary recipient mice (data not shown). These results indicate that continuous administration of dasatinib could prevent these residual cells from developing into fatal B-ALL, although this compound did not completely kill the residual leukemic cells (Fig. 4*A*).

We identified the cell types of these residual GFP^+ cells as $B220^+/CD43^+$ and $B220^+/CD43^-$ pro-/pre-B cells (Fig. 4*B*), and these progenitor leukemic cells may have acquired self-renewal capacity and function as B-ALL stem cells. To test this hypothesis, we sorted by FACS the $B220^+/CD19^+/GFP^+$ cells from the BM of B-ALL mice, followed by transplantation of the cells into recipient mice. These mice developed B-ALL after 2 months, and leukemic cells in peripheral blood were $CD19^+/CD43^+$ pro-B cells (Fig. 4*C*). We conclude that $CD19^+/B220^+/CD43^+$ pro-B cells expressing BCR-ABL can function as B-ALL stem cells. To support this conclusion, we transferred purified BCR-ABL-expressing $CD19^+/B220^+/CD43^+$ pro-B cells into recipient mice; these cells induced leukemia and had potential to differentiate (data not shown).

Dasatinib Significantly Prolongs Survival of CML Mice but Does Not Eradicate CML Stem Cells. Dasatinib is very effective in controlling B-ALL (Fig. 3*A*). We tested whether dasatinib also is effective at treating CML in mice. CML mice treated with dasatinib lived significantly longer than those treated with imatinib (Fig. 5*A*), which correlated with significantly lower numbers of BCR-ABL-expressing leukemic cells in peripheral blood (Fig. 5*B*) compared with placebo- or imatinib-treated mice. However, all dasatinib-treated CML mice eventually died of this disease (Fig. 5*A*), indicating that, like imatinib (21), this drug may not eradicate leukemic stem cells in CML mice. Because CML in mice originates from multilineage repopulating cells (14), we tested whether dasatinib kills BCR-ABL-expressing hematopoietic stem cells (HSCs) *in vivo*. We used BALB/c mice to induce CML because we used this strain in our therapeutic experiments in this study (Figs. 3*A* and 5*A*). We treated CML mice with a placebo, imatinib, or dasatinib for 14 days, starting from day 8 after CML induction by BCR-ABL, and found that BCR-ABL-expressing HSCs (GFP^+CD34^-c-*Kit*$^+Hoe^-$) existed in the side population (22) of BM cells from the imatinib- or dasatinib-treated CML mice (Fig. 5*C*). This observation indicates that neither imatinib nor dasatinib completely eradicates BCR-ABL-expressing HSCs, suggesting that neither drug will cure CML and that targeting at least one additional component of BCR-ABL-expressing HSCs is required for curing the disease. Because analysis of HSCs in side populations for the existence of the stem cells is not quantitative, we further analyzed BCR-ABL-expressing HSCs in dasatinib-treated CML mice (in B6 background) by identifying the GFP^+Lin^-c-*kit*$^+Sca\text{-}1^+$ population. Compared with placebo-treated mice, dasatinib reduced the numbers of BCR-ABL-expressing HSCs but failed to eradicate these cells completely in CML mice (data not shown), consistent with the finding using BALB/c mice (Fig. 5*C*). This biological effect on BCR-ABL-expressing HSCs does not support the possibility that inability of dasatinib to completely eradicate BCR-ABL-expressing HSCs may be attributed to the failure of dasatinib to access the stem cells, because we detected inhibition of intracellular BCR-ABL phosphorylation by dasatinib in the stem cells (Fig. 5*D*). The inability of dasatinib to cure CML mice is not attributed to the appearance of BCR-ABL-T315I clone in the mice, because CML mice treated with dasatinib for ≈3 months contained >40% of $GFP^+Gr\text{-}1^+$ cells, among which there were large numbers of stem cells (Fig. 5*E*), and sequencing analysis of isolated genomic DNA from BM cells

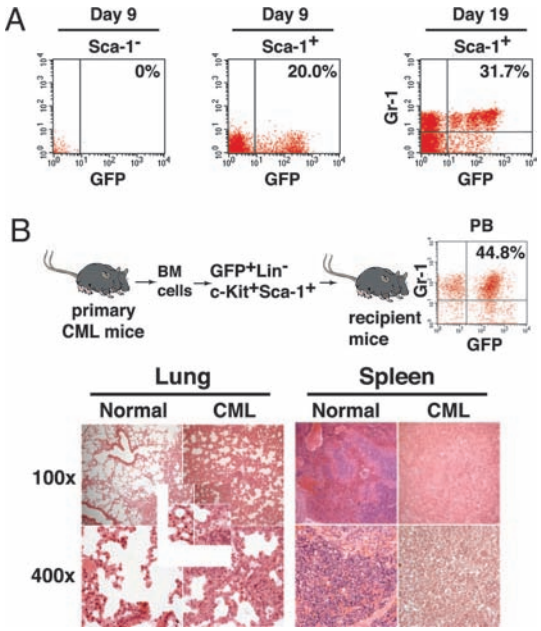

Fig. 6. Identification of BM cell populations that function as CML stem cells. (*A*) BCR-ABL-transduced BM cells from B6 mice were sorted by Sca-1 MACS columns (Miltenyi Biotec, Gladbach, Germany), followed by transferring a Sca-1$^-$ or Sca-1$^+$ population into B6 mice (1×10^5 cells per mouse; four mice per cell population group) to induce CML. GFP$^+$ myeloid cells (Gr-1$^+$) in peripheral blood (PB) of the mice were examined at days 9 and 19 after the induction of leukemia. All mice receiving the Sca-1$^+$ population died of CML by day 42. (*B*) BCR-ABL-expressing HSCs function as CML stem cells. BM cells from CML mice in B6 background were sorted by FACS for BCR-ABL-expressing HSCs (GFP$^+$Lin$^-$c-*kit*$^+$Sca-1$^+$), followed by transfer into lethally irradiated B6 mice (2×10^4 cells per mouse). GFP$^+$ myeloid cells (Gr-1$^+$) were detected in peripheral blood. In contrast to the normal control mice, CML mice showed complete infiltration of the lungs with myeloid leukemic cells and complete disruption of follicular architecture of the spleen by infiltrating leukemic cells.

of these mice did not show T315I mutation in the BCR-ABL kinase domain (data not shown). The failure of imatinib to eradicate BCR-ABL-expressing HSCs is not related to c-*kit* function, because both imatinib and dasatinib inhibit c-*kit* (23). These results suggest that inhibition of BCR-ABL kinase activity alone is insufficient to eradicate CML stem cells.

To identify CML stem cells, we tested whether BCR-ABL-expressing HSCs function as the stem cells. We first sorted C57BL/6 (B6) BM cells transduced with BCR-ABL retrovirus into two separate populations: Sca-1$^-$ and Sca-1$^+$. These two populations of cells were transferred, respectively, into B6 mice. Only the mice receiving BCR-ABL-transduced Sca-1$^+$ cells developed and died of CML, diagnosed by detecting GFP$^+$ myeloid cells (Gr-1$^+$) in the peripheral blood of the mice (Fig. 6*A*). This result suggests that early BM progenitors contain CML stem cells. To narrow down the specific cell lineages that function as CML stem cells, HSCs (Lin$^-$c-*kit*$^+$Sca-1$^+$) were sorted out from BM cells transduced with BCR-ABL retrovirus, followed by transfer into recipient mice. The mice developed and died of CML (data not shown). To confirm definitively that BCR-ABL-expressing HSCs are CML stem cells, we isolated BM cells from primary CML mice and sorted out the BCR-ABL-expressing HSCs (GFP$^+$Lin$^-$c-*Kit*$^+$Sca-1$^+$) by FACS. The sorted cells were transferred into recipient mice, and the mice developed and died of CML (Fig. 6*B*), indicating that BCR-ABL expressing HSCs function as CML stem cells.

Discussion

Our findings provide evidence that inhibition solely of BCR-ABL kinase activity is effective at treating Ph$^+$ B-ALL and CML in mice but is not sufficient to achieve complete control of these two types of leukemia. This failure is partially caused by BCR-ABL activation of signaling pathways, such as SRC kinases, that are not inhibited by imatinib and essential to leukemia development. Sustained activation of these pathways would allow leukemic cells to survive treatment with compounds that inhibit only BCR-ABL kinase activity until the emergence of drug resistance. Simultaneous targeting of these pathways and BCR-ABL kinase activity would provide a vastly improved therapeutic approach to chemotherapy of Ph$^+$ leukemias. This strategy is in contrast to a general idea that complete and sole inhibition of BCR-ABL kinase activity would completely inhibit BCR-ABL functions.

SRC kinases play a critical role in the development of BCR-ABL-induced B-ALL (5). Sole inhibition of BCR-ABL kinase activity with kinase inhibitors will not shut down the SRC pathway, suggesting the existence of a BCR-ABL kinase activity-independent pathway. This pathway would help leukemic cells survive treatment and eventually allow resistant BCR-ABL-T315I clones to grow out. BCR-ABL-activated SRC kinases alone may not transform B-lymphoid cells efficiently, but they are sufficient to maintain survival and stimulate proliferation of the leukemic cells. The next generation of BCR-ABL kinase inhibitors aims at increasing drug potency or overriding imatinib resistance caused by kinase domain point mutations, including BCR-ABL-T315I. However, to achieve a durable therapeutic effect in patients with Ph$^+$ B-ALL and lymphoid blast crisis, SRC kinases must be targeted. Our study suggests that targeting SRC kinases with dasatinib may delay transition of CML chronic phase to blast crisis and may be effective in treating acute lymphoid leukemia with compromised tumor suppressor function, providing a rationale for the early and continuous use of dasatinib in chronic-phase CML patients. The parallel results attained with the triple SRC kinase knockout cells and dasatinib demonstrate the role of certain pathways involving SRC kinases in the more advanced phases of CML and suggest that targeting SRC kinases and BCR-ABL with dasatinib may be an effective therapy for preventing transition of patients with chronic-phase CML to lymphoid blast crisis and for management of patients with advanced lymphoid leukemia. In fact, dasatinib is effective in treating Ph$^+$ B-ALL patients (24). If the BCR-ABL-T315I mutation is present in leukemic cells, this BCR-ABL-driven disease cannot be averted by dasatinib. However, dasatinib treatment may lead to long-term remission of B-ALL if the T315I mutation is absent from the leukemic cell population. The weak therapeutic effect of imatinib is unlikely to be attributed to an insufficient dose of imatinib, because the 100 mg/kg dose of imatinib administered in the mice inhibited BCR-ABL kinase activity *in vivo* significantly and to a similar degree compared with dasatinib.

Although dasatinib does not kill leukemic stem cells completely in B-ALL mice, targeting SRC kinases and perhaps other as-yet-unidentified signaling molecules could help achieve long-term control of the disease. Curative drug therapy of B-ALL would require targeting not only BCR-ABL kinase activity and SRC-dependent pathways, but also quiescent primitive leukemic cells (25). We identified pro-B leukemic cells as stem cells for B-ALL in mice. The rapid and striking hematologic response of B-ALL mice to dasatinib suggests that these pro-B progenitors with acquired self-renewal capacity are the major source of highly proliferating B-lymphoid leukemic cells in B-ALL mice and that complete inhibition of growth of this leukemic population could achieve long-term survival of B-ALL mice. Moreover, inhibiting the expansion of this population would reduce the frequency of the appearance of resistance mutations. It will be critical to assess whether BCR-ABL-expressing pro-B cells serve as stem cells

in patients with Ph^+ B-ALL or lymphoid blast crisis CML, because BCR-ABL can convert progenitors to leukemic stem cells (26). We also identified CML stem cells in mice as $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ cells, and these cells are insensitive to inhibition by imatinib and dasatinib. Thus, identification of unknown pathways in CML stem cells will be critical for developing curative therapies for the disease.

Methods

Cell Lines. The BaF/3 pre-B and ENU cell lines were grown in RPMI medium 1640 containing 10% FCS, 10% WEHI medium, and 50 μM 2-mercaptoethanol.

Whitlock–Witte Culture. BM cells were transduced with the BCR-ABL retrovirus and cultured as described previously (5).

Antibodies and Western Blot Analysis. Antibodies against phosphotyrosine, c-ABL, CrkL, β-actin, and the SRC kinases were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against c-Abl-Y412, phospho-CrkL, and SRC-Y416 were purchased from Cell Signaling Technology (Danvers, MA). Protein lysates were prepared by lysing cells in RIPA buffer, and immunoprecipitation and Western blotting were carried out as described previously (27).

BM Transduction/Transplantation. The retroviral vector MSCV-IRES-eGFP carrying the BCR-ABL cDNA was used to make virus stock as described previously (14). Four- to 10-week-old wild-type BABL/c or C57BL/6 (The Jackson Laboratory) and homozygous SRC triple gene knockout ($Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$) mice (5) were used for leukemogenesis experiments (14, 28).

Flow Cytometry. Hematopoietic cells were collected from the diseased mice and analyzed by FACS analysis as described previously (5).

Drug Treatment. Dasatinib was dissolved in 80 mM citric acid (pH 2.1) to make 10 mg/ml stock solution and then diluted to 1 mg/ml with 80 mM citric acid (pH 3.1) for use. Imatinib was dissolved in water directly at a concentration of 10 mg/ml. The drugs were given orally in a volume of <0.5 ml by gavage twice a day, at 10 mg per kilogram of body weight per dose for dasatinib and 100 mg per kilogram of body weight for imatinib, beginning at 8 days after BM transplantation and continuing until the morbidity or death of the leukemic mice.

We thank David Serreze, Barbara Tennent, and Stephen Sampson for critical reading of the manuscript and Patricia Cherry for secretarial assistance. This work was supported by grants from the Linda Tallen and David Paul Kane Educational and Research Foundation, the Irving A. Hansen Foundation, the U.S. Department of Defense, and the Maine Cancer Foundation and by National Cancer Institute Grant CA114199 (to S.L.).

1. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, *et al*. (2001) *N Engl J Med* 344:1031–1037.
2. Graham SM, Jorgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, Holyoake TL (2002) *Blood* 99:319–325.
3. Marley SB, Deininger MW, Davidson RJ, Goldman JM, Gordon MY (2000) *Exp Hematol* 28:551–557.
4. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL (2001) *Science* 293:876–880.
5. Hu Y, Liu Y, Pelletier S, Buchdunger E, Warmuth M, Fabbro D, Hallek M, Van Etten RA, Li S (2004) *Nat Genet* 36:453–461.
6. Wolff NC, Ilaria RL, Jr (2001) *Blood* 98:2808–2816.
7. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL (2004) *Science* 305:399–401.
8. O'Hare T, Pollock R, Stoffregen EP, Keats JA, Abdullah OM, Moseson EM, Rivera VM, Tang H, Metcalf CA, III, Bohacek RS, *et al*. (2004) *Blood* 104:2532–2539.
9. Weisberg E, Manley PW, Breitenstein W, Bruggen J, Cowan-Jacob SW, Ray A, Huntly B, Fabbro D, Fendrich G, Hall-Meyers E, *et al*. (2005) *Cancer Cell* 7:129–141.
10. Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M (2001) *N Engl J Med* 344:1038–1042.
11. Donato NJ, Wu JY, Stapley J, Gallick G, Lin H, Arlinghaus R, Talpaz M (2003) *Blood* 101:690–698.
12. Roumiantsev S, Shah NP, Gorre ME, Nicoll J, Brasher BB, Sawyers CL, Van Etten RA (2002) *Proc Natl Acad Sci USA* 99:10700–10705.
13. Warmuth M, Simon N, Mitina O, Mathes R, Fabbro D, Manley PW, Buchdunger E, Forster K, Moarefi I, Hallek M (2003) *Blood* 101:664–672.
14. Li S, Ilaria RL, Jr, Million RP, Daley GQ, Van Etten RA (1999) *J Exp Med* 189:1399–1412.
15. Wilson MB, Schreiner SJ, Choi HJ, Kamens J, Smithgall TE (2002) *Oncogene* 21:8075–8088.
16. Pear WS, Miller JP, Xu L, Pui JC, Soffer B, Quackenbush RC, Pendergast AM, Bronson R, Aster JC, Scott ML, *et al*. (1998) *Blood* 92:3780–3792.
17. Sill H, Goldman JM, Cross NC (1995) *Blood* 85:2013–2016.
18. Towatari M, Adachi K, Kato H, Saito H (1991) *Blood* 78:2178–2181.
19. Feinstein E, Cimino G, Gale RP, Alimena G, Berthier R, Kishi K, Goldman J, Zaccaria A, Berrebi A, Canaani E (1991) *Proc Natl Acad Sci USA* 88:6293–6297.
20. Williams RT, Roussel MF, Sherr CJ (2006) *Proc Natl Acad Sci USA* 103:6688–6693.
21. Holtz MS, Slovak ML, Zhang F, Sawyers CL, Forman SJ, Bhatia R (2002) *Blood* 99:3792–3800.
22. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) *J Exp Med* 183:1797–1806.
23. Heinrich MC, Blanke CD, Druker BJ, Corless CL (2002) *J Clin Oncol* 20:1692–16703.
24. Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, Paquette R, Cortes J, O'Brien S, Nicaise C, Bleickardt E, *et al*. (2006) *N Engl J Med* 354:2531–2541.
25. Elrick LJ, Jorgensen HG, Mountford JC, Holyoake TL (2005) *Blood* 105:1862–1866.
26. Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, Gotlib J, Li K, Manz MG, Keating A, *et al*. (2004) *N Engl J Med* 351:657–667.
27. Li S, Couvillon AD, Brasher BB, Van Etten RA (2001) *EMBO J* 20:6793–6804.
28. Roumiantsev S, de Aos IE, Varticovski L, Ilaria RL, Van Etten RA (2001) *Blood* 97:4–13.
29. Hunter T (1987) *Cell* 49:1–4.

Inhibition of heat shock protein 90 prolongs survival of mice with BCR-ABL-T315I–induced leukemia and suppresses leukemic stem cells

Cong Peng,[1] Julia Brain,[2] Yiguo Hu,[1] Ami Goodrich,[1] Linghong Kong,[1] David Grayzel,[2] Roger Pak,[2] Margaret Read,[2] and Shaoguang Li[1]

[1]The Jackson Laboratory, Bar Harbor, ME; [2]Infinity Pharmaceuticals, Cambridge, MA

Development of kinase domain mutations is a major drug-resistance mechanism for tyrosine kinase inhibitors (TKIs) in cancer therapy. A particularly challenging example is found in Philadelphia chromosome–positive chronic myelogenous leukemia (CML) where all available kinase inhibitors in clinic are ineffective against the BCR-ABL mutant, T315I. As an alternative approach to kinase inhibition, an orally administered heat shock protein 90 (Hsp90) inhibitor, IPI-504, was evaluated in a murine model of CML. Treatment with IPI-504 resulted in BCR-ABL protein degradation, decreased numbers of leukemia stem cells, and prolonged survival of leukemic mice bearing the T315I mutation. Hsp90 inhibition more potently suppressed T315I-expressing leukemia clones relative to the wild-type (WT) clones in mice. Combination treatment with IPI-504 and imatinib was more effective than either treatment alone in prolonging survival of mice simultaneously bearing both WT and T315I leukemic cells. These results provide a rationale for use of an Hsp90 inhibitor as a first-line treatment in CML by inhibiting leukemia stem cells and preventing the emergence of imatinib-resistant clones in patients. Rather than inhibiting kinase activity, elimination of mutant kinases provides a new therapeutic strategy for treating BCR-ABL–induced leukemia as well as other cancers resistant to treatment with tyrosine kinase inhibitors. (Blood. 2007; 110:678-685)

Introduction

The human Philadelphia chromosome (Ph) arises from a translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)].[1] The resulting chimeric *BCR-ABL* oncogene encodes a constitutively activated, oncogenic tyrosine kinase that induces chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL). The BCR-ABL TKI, imatinib mesylate, induces a complete hematologic and cytogenetic response in the majority of chronic-phase CML patients,[2] but is unable to completely eradicate BCR-ABL–expressing leukemic cells,[3,4] suggesting that leukemia stem cells are not eliminated. Over time, patients frequently become drug resistant and develop progressive disease despite continued treatment.[5-11] Resistance is predominantly due to emergence of kinase domain mutations. Three newly developed BCR-ABL kinase inhibitors—dasatinib,[12] AP23464,[13] and AMN107[14]—inhibit most of imatinib-resistant BCR-ABL mutants at biochemical and cellular levels, but are ineffective against the BCR-ABL-T315I mutant.[15,16] New approaches are needed to treat drug-resistant forms of CML as well as BCR-ABL–induced B-ALL, a leukemia that does not respond well to available TKIs.[15,16]

Heat shock protein 90 (Hsp90) is a highly conserved, constitutively expressed molecular chaperone that facilitates folding of client proteins such as BCR-ABL, and affects the stability of these proteins.[17-21] When BCR-ABL contains resistance-conferring mutations, it becomes even more dependent on Hsp90 in vitro.[20] We therefore evaluated the therapeutic effect of Hsp90 inhibition by using a novel water-soluble inhibitor, IPI-504,[22] in drug-resistant animal models of leukemia induced by BCR-ABL-WT and T315I.

Materials and methods

Cell lines

The 32D myeloid cell line was grown in RPMI 1640 medium containing 10% FCS and 10% WEHI medium. The BaF/3 pre-B-cell line was grown in RPMI 1640 medium containing 10% FCS, 10% WEHI medium, and 50 μM 2-mercaptoethanol. To generate the BCR-ABL–expressing 32D or BaF/3 line, the cells were transduced with the BCR-ABL-WT- or BCR-ABL-T315I-IRES-GFP-MSCV retrovirus, and the BCR-ABL–expressing cells were selected by GFP sorting by fluorescence-activated cell sorter (FACS).

Histology

The lungs from the placebo- or drug-treated mice were fixed in Bouin fixative (Fisher Scientific, Pittsburgh, PA) for 24 hours at room temperature, followed by an overnight rinse in water. Ten-μm sections were stained with hematoxylin and eosin (H&E) and observed by a model DMRE compound microscope (Leica, Heidelberg, Germany). All sections were imaged with a 2.5 × PH1 objective (NPLan, NA 0.25) and 10 × PH1 objective (NPLan, NA 0.40). All images were imported into MetaMorph software (Molecular Devices, Downingtown, PA) as a series of tagged image files. All images were then constructed in Adobe Photoshop 6.0 (Adobe, San Jose, CA).

Antibodies and Western blot analysis

Antibodies against c-ABL, Hsp90, Hsp70, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein lysates were prepared by lysing cells in radioimmunoprecipitation (RIPA) buffer, and immunoprecipitation and Western blotting were carried out as described previously.[23]

Submitted October 25, 2006; accepted March 21, 2007. Prepublished online as *Blood* First Edition paper, March 29, 2007; DOI 10.1182/blood-2006-10-054098.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Bone marrow transduction/transplantation

The retroviral vector MSCV-IRES-eGFP[24] carrying the p210 *BCR-ABL* cDNA was used to make high-titer, helper-free, replication-defective ecotropic virus stock by transient transfection of 293T cells using the kat system,[25] as previously described.[26] Six- to 10-week-old wild-type BABL/c or C57BL/6 mice (The Jackson Laboratory) were used for leukemogenesis experiments. Induction of CML[26] and B-ALL[26,27] was as described previously. Briefly, to model CML, bone marrow from 5-FU–treated (200 mg/kg) donor mice was transduced twice with *BCR-ABL* retrovirus by cosedentation in the presence of IL-3, IL-6, and SCF. To model B-ALL, bone marrow from non–5-FU–treated donors was transduced without cytokines. Wild-type recipient mice were prepared by 900 cGy (for BABL/c) or 1150 cGy (for C57BL/6) gamma irradiation and a dose of 0.5×10^6 (CML) or 1.0×10^6 (B-ALL) cells transplanted via tail vein injection. Diseased mice were analyzed by histopathological and biochemical analyses as described previously.[26]

Flow cytometry

Hematopoietic cells were collected from peripheral blood and bone marrow of the diseased mice, and red blood cells were lysed with NH_4Cl red blood cell lysis buffer (pH 7.4). The cells were washed with PBS, and stained with B220-PE for B cells, Gr1-APC for neutrophils, and Sca1-APC/c-kit-PE for hematopoietic stem cells. After staining, the cells were washed once with PBS and subjected to FACS analysis.

Culture of leukemia stem cells

Bone marrow cells isolated from CML mice were cultured in vitro in the presence of stemspan SFEM, SCF, IGF-2, TPO, heparin, and α-FGF as reported previously for culture of hematopoietic stem cells.[28,29]

Drug treatment

IPI-504 was dissolved in a solution containing 50 mM citrate, 50 mM ascorbate, 2.44 mM EDTA, pH 3.3. Imatinib was dissolved in water. The drugs were given orally in a volume of less than 0.5 mL by gavage (50 or 100 mg/kg, every other day for IPI-504, and 100 mg/kg, twice a day for imatinib) beginning at 8 days after bone marrow transplantation, and continuing until the morbidity or death of the leukemic mice. Placebo is a solution containing 50 mM citrate, 50 mM ascorbate, 2.44 mM EDTA, pH 3.3.

Results

Inhibition of Hsp90 by IPI-504 causes BCR-ABL protein degradation

IPI-504 is the hydroquinone hydrochloride derivative of the well-described Hsp90 inhibitor, 17-AAG; the chemical structure of IPI-504 is shown in Figure 1A. To examine the effects of IPI-504 on stability of BCR-ABL protein and to test whether the degradation of BCR-ABL protein is initiated through IPI-504–induced disassociation of BCR-ABL from Hsp90, T315I-32D myeloid cells were treated with IPI-504 for 30 minutes and 4 hours, respectively. Hsp90 protein was immunoprecipitated and Hsp90-associated BCR-ABL protein was assessed. IPI-504 induced complete disassociation of BCR-ABL and Hsp90 within 30 minutes, followed by loss of BCR-ABL protein at 4 hours (Figure 1B). These results demonstrate that BCR-ABL protein is degraded after inhibition of Hsp90 by IPI-504 and this degradation occurs after disassociation of BCR-ABL from Hsp90. To further demonstrate that IPI-504 mediates the degradation of BCR-ABL through the proteasome, T315I-32D myeloid cells were treated with IPI-504 alone for up to 8 hours or with both IPI-504 and a proteasome inhibitor PS-341[30,31] that should inhibit BCR-ABL degradation caused by IPI-504.

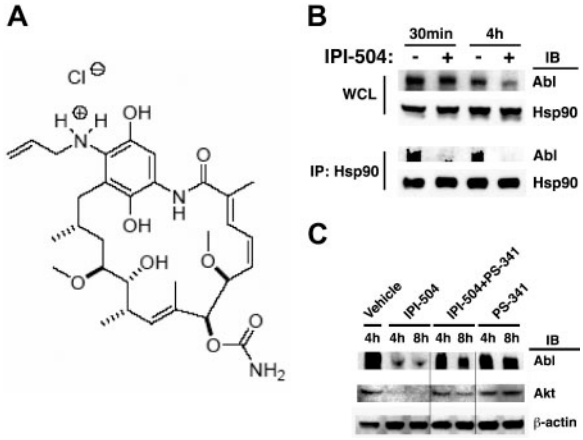

Figure 1. Inhibition of Hsp90 by IPI-504 causes BCR-ABL protein degradation. (A) Structure of IPI-504. (B) IPI-504–induced disassociation of BCR-ABL and Hsp90, and subsequent degradation of BCR-ABL protein. BCR-ABL-T315I–expressing 32D cells were treated with IPI-504 (2 μM) for 30 minutes and 4 hours, respectively. Protein lysates were analyzed by Western blotting using antibodies indicated. WCL indicates whole cell lysate; IP, immunoprecipitation; and IB, immunoblotting. (C) The proteasome inhibitor PS-341 restored IPI-504–mediated depletion of BCR-ABL protein. BCR-ABL-T315I–expressing 32D cells were treated with IPI-504 (2 μM) alone or IPI-504 plus PS-341 (100 nM) for 4 or 8 hours, respectively. Protein lysates were analyzed by Western blotting using antibodies indicated. The well-described Hsp90 client, Akt, was evaluated as a positive control. Note that the cells were pretreated with PS-341 for 30 minutes prior to the cotreatment with IPI-504 and PS-341. The black lines indicate that the lanes that were not adjacent on the same original Western blotting gel were brought together to generate this figure.

PS-341 restored IPI-504–mediated depletion of BCR-ABL protein (Figure 1C).

Hsp90 is a therapeutic target for BCR-ABL–induced CML

An investigation of whether Hsp90 is an effective target for the treatment of CML in vivo was conducted in the bone marrow transplantation (BMT) mouse model of CML, in which bone marrow cells from BALB/c donor mice pretreated with 5-fluorouracil (5-FU) and transduced with *BCR-ABL* results in development of CML in BALB/c recipient mice.[26] Mice with WT or T315I-transduced bone marrow from 5-FU–treated WT BALB/c donor mice were treated with a placebo, the Hsp90 inhibitor IPI-504, or imatinib alone, or the 2 agents in combination. All placebo-treated mice developed and died of CML within 3 weeks after BMT (Figure 2A). As expected, imatinib treatment was effective in treating WT-induced CML but not CML induced by T315I (Figure 2A). In a dose-dependent manner, treatment with IPI-504 alone significantly prolonged survival of mice with WT CML, but even more markedly prolonged survival of mice with T315I-induced CML (Figure 2A, $P < .001$). Inhibition of Hsp90 by IPI-504 appears to be more effective in treating CML induced by T315I than by WT BCR-ABL, consistent with results in Figure 1A and in line with previously reported results with the Hsp90 inhibitor, 17-AAG.[20] In both cases, inhibition of Hsp90 results in degradation of mutant BCR-ABL more readily than WT. Treatment of mice with WT CML with both IPI-504 and imatinib was slightly more effective (but statistically insignificant) than with imatinib alone in prolonging survival of the mice (Figure 2A), while treatment of mice with BCR-ABL-T315I–induced CML with these 2 drugs did not further prolong survival of the mice compared with the mice treated with IPI-504 alone (Figure 2A). Prolonged survival of IPI-504–treated CML mice correlated with decreased peripheral blood BCR-ABL–expressing (GFP-positive) leukemia cells during therapy (Figure 2B, $P < .001$) and less splenomegaly at necropsy

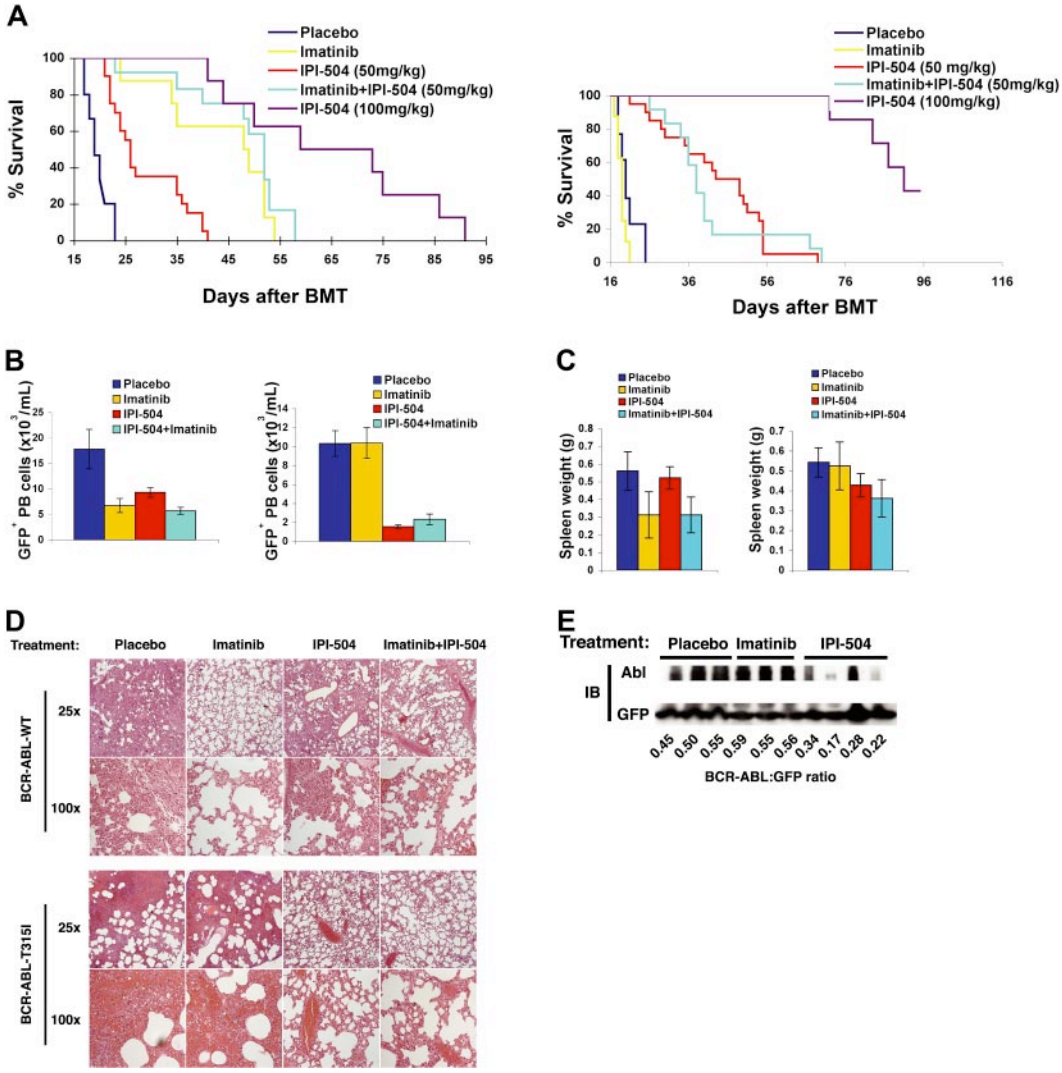

Figure 2. Hsp90 is a therapeutic target for CML induced by either BCR-ABL-WT or BCR-ABL-T315I. (A) Treatment with the Hsp90 inhibitor IPI-504 prolonged survival of CML mice. Mice with BCR-ABL-WT (left panel)– or BCR-ABL-T315I (right panel)–induced CML were treated with placebo (n = 15 for BCR-ABL-WT; n = 13 for BCR-ABL-T315I), imatinib (100 mg/kg, twice a day by gavage) (n = 8 for both BCR-ABL-WT and -T315I), IPI-504 (50 mg/kg, once every 2 days by gavage) (n = 20 for both BCR-ABL-WT and BCR-ABL-T315I), IPI-504 (100 mg/kg, once every 2 days by gavage) (n = 8 for both BCR-ABL-WT; n = 7 for BCR-ABL-T315I), and imatinib + IPI-504 (n = 12 for both BCR-ABL-WT and -T315I), respectively, beginning at day 8 after transplantation. The IPI-504–treated mice with BCR-ABL-T315I–induced CML lived longer than those with BCR-ABL-WT–induced CML (comparing between left and right panels). (B) Flow cytometric evaluation of the leukemic process in IPI-504– or imatinib-treated CML mice. The number of circulating leukemic cells (calculated as percentage of Gr-1$^+$ GFP$^+$ cells × white blood cell count) in mice with BCR-ABL-WT (left panel)– or BCR-ABL-T315I (right panel)–induced CML treated with placebo, imatinib, IPI-504, or the combination of imatinib and IPI-504 was determined on day 14 after transplantation. (C) Spleen weights of CML mice treated with placebo, imatinib, IPI-504, and combination of imatinib and IPI-504. (Left panel) BCR-ABL-WT. (Right panel) BCR-ABL-T315I. (D) Photomicrographs of hematoxylin and eosin–stained lung sections from drug-treated mice at day 14 after transplantation. (E) Western blot analysis of spleen-cell lysates for degradation of BCR-ABL in IPI-504–treated CML mice. IB indicates immunoblot.

(Figure 2C). As lung hemorrhage caused by infiltration of mature myeloid leukemia cells is a major cause of death of CML mice,[26] we further evaluated the therapeutic effect of IPI-504 on CML by examining the severity of lung hemorrhages at day 15 after BMT. Compared with placebo-treated mice, fewer hemorrhages were observed in the lungs of IPI-504–treated mice with BCR-ABL-T315I–induced CML (Figure 2D). Western blot analysis of spleen-cell lysates from the treated CML mice showed that IPI-504 reduced the levels of BCR-ABL protein in CML mice (Figure 2E).

Hsp90 is also a therapeutic target for B-ALL induced by BCR-ABL-T315I

CML often initiates in a chronic phase and eventually progresses to a terminal blastic phase, in which either acute myeloid or acute B-lymphoid leukemia develops.[32] Some Ph$^+$ leukemia patients have B-ALL as their first clinical appearance. B-ALL is similar pathologically to acute B-lymphoid leukemia in the blastic phase of CML. Notably, both forms of acute leukemia do not respond well to available BCR-ABL kinase inhibitors.[15,16] To model B-ALL in mice, BCR-ABL–transduced bone marrow cells from donor mice that are not pretreated with 5-FU are transplanted into BALB/c mice.[26,33] In this model, the malignant pre-B cells express the cell surface markers B220 and CD19, and phenotypically resemble de novo Ph$^+$ B-ALL and lymphoid blast crisis of CML.[26,27] To determine whether inhibition of Hsp90 is effective in treating WT or T315I-induced B-ALL, these mice were treated with a placebo, IPI-504 alone, imatinib alone, or the 2 agents in combination (Figure 3). All placebo-treated recipients of WT or T315I-transduced bone marrow developed and died of B-ALL within 5 to 6 weeks after BMT (Figure 3A). IPI-504 treatment did not prolong

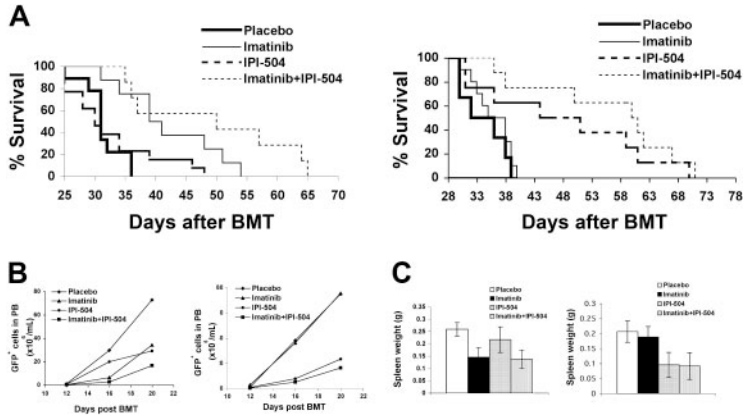

Figure 3. Hsp90 is a therapeutic target for B-ALL induced by BCR-ABL-T315I. (A) Treatment with the Hsp90 inhibitor IPI-504 prolonged survival of mice with B-ALL induced by BCR-ABL-T315I (right panel) but not by BCR-ABL-WT (left panel). B-ALL mice treated with a placebo (n = 9 for BCR-ABL-WT; n = 8 for BCR-ABL-T315I), imatinib (n = 8 for BCR-ABL-WT; n = 10 for BCR-ABL-T315I), IPI-504 (n = 13 for BCR-ABL-WT; n = 8 for BCR-ABL-T315I), and combination of imatinib and IPI-504 (n = 10 for BCR-ABL-WT; n = 8 for BCR-ABL-T315I). (B) Flow cytometric evaluation of the leukemic process in IPI-504– or imatinib-treated mice with B-ALL induced by BCR-ABL-WT (left panel) or BCR-ABL-T315I (right panel). The number of circulating leukemic cells (calculated as percentage of $B220^+$ GFP^+ cells × white blood cell count) in B-ALL mice treated with placebo, imatinib, IPI-504, or the combination of imatinib and IPI-504 was determined on days 11, 14, and 17 after transplantation. (C) Spleen weights of B-ALL mice treated with placebo, imatinib, IPI-504, and combination of imatinib and IPI-504. (Left panel) BCR-ABL-WT. (Right panel) BCR-ABL-T315I.

survival of mice with BCR-ABL-WT–induced B-ALL (Figure 3A), in contrast to its therapeutic effect on CML induced by BCR-ABL-WT (Figure 2A). Given the dose response seen in the CML study and significant improvement in survival when the dose of IPI-504 is increased from 50 to 100 mg, a similar increase in dose may be needed in B-ALL. However, similar to the effect seen in CML (Figure 2A), IPI-504 treatment significantly prolonged survival of mice with T315I- B-ALL (Figure 3A, $P < .001$). Prolonged survival of IPI-504–treated B-ALL mice correlated with decreased numbers of peripheral blood BCR-ABL–expressing leukemia cells and spleen weights during therapy (Figure 3B-C, $P < .001$). Once again, inhibition of Hsp90 by IPI-504 is more effective against tumor cells bearing T315 than BCR-ABL-WT.

Hsp90 inhibition has differential effects on BCR-ABL degradation and Hsp70 induction in myeloid and lymphoid cells in vitro

To investigate why inhibition of Hsp90 is more effective in treating CML than B-ALL (Figures 2-3), we compared the effects of treatment with IPI-504 on BCR-ABL-WT or BCR-ABL-T315I at protein level in a mouse myeloid cell line (32D) and a mouse lymphoid cell line (BaF/3) (Figure 4). BCR-ABL–expressing 32D and BaF/3 cells were treated with different concentrations of IPI-504. After treatment, levels of BCR-ABL-WT protein were dramatically decreased in 32D cells (Figure 4A), but only slightly in BaF/3 cells (Figure 4B). Compared with BCR-ABL-WT, BCR-ABL-T315I was more sensitive to IPI-504–induced degradation in both 32D and BaF/3 cells, but levels of BCR-ABL protein were decreased much more markedly in 32D cells than in BaF/3 cells (Figure 4A-B). These results indicate that inhibition of Hsp90 by IPI-504 affects BCR-ABL stability more strongly in myeloid cells than in lymphoid cells. It has been shown that the Hsp90 antagonists geldanamycin and 17-AAG alter chaperone association of Hsp90 with BCR-ABL and facilitate binding of BCR-ABL to heat shock protein 70 (hsp70), resulting in degradation of BCR-ABL by the proteasome.[19,34-36] Recent studies have shown that Hsp70 plays a positive role in BCR-ABL–mediated resistance to apoptosis.[37,38] If Hsp70 plays a role in decreased sensitivity of B-ALL than CML to IPI-504 treatment, we expect that after IPI-504 treatment, Hsp70 would be induced to a much higher level in BCR-ABL–expressing lymphoid cells than in myeloid cells. However, an increase in intracellular Hsp70 levels was observed in IPI-504–treated BCR-ABL–expressing 32D but not Ba/F3 cells (Figure 4A-B). This observation is consistent with our in vivo observation in cells from CML and B-ALL mice, which showed that the level of Hsp70 in leukemic cells from IPI-504–treated CML mice is higher than that in leukemic cells from B-ALL mice (Figure 4C-D). Thus, Hsp70 is not an explanation for the decreased sensitivity of B-ALL compared with CML upon IPI-504 treatment (Figures 2-3).

Inhibition of Hsp90 suppresses CML stem cells

In the BMT CML model, imatinib prolongs survival of mice with BCR-ABL–induced CML,[33,39] but does not stop progression of the disease,[33] partially due to the inability of imatinib to completely eradicate leukemia stem cells.[40] Hematopoietic stem cells (HSCs) have been identified in the CML model by showing that the $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ population is sufficient to confer leukemia in recipient mice.[40] To investigate whether inhibition of Hsp90 has an

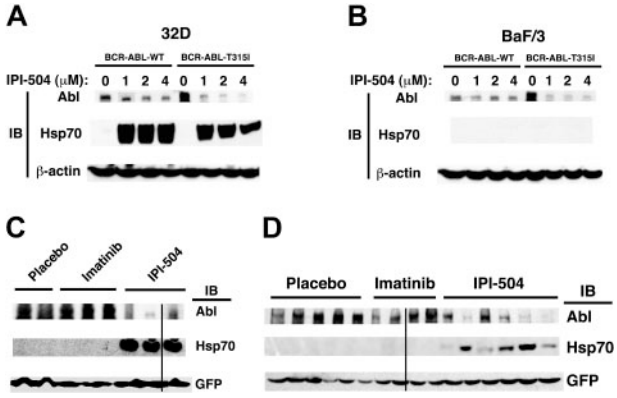

Figure 4. Hsp90 inhibition has differential effects on BCR-ABL degradation and Hsp70 induction in myeloid and lymphoid cells in vitro and in vivo. (A) In 32D cells, IPI-504–induced degradation of BCR-ABL-T315I was greater than that of BCR-ABL-WT. BCR-ABL-WT– or BCR-ABL-T315I–expressing 32D cells were treated with different concentrations of IPI-504 for 12 hours. Protein lysates were analyzed by Western blotting using antibodies indicated. (B) In Ba/F3 cells, IPI-504 induced significant degradation of BCR-ABL-T315I but not BCR-ABL-WT. BCR-ABL-WT– or BCR-ABL-T315I–expressing 32D cells were treated with different concentrations of IPI-504 for 12 hours. Protein lysates were analyzed by Western blotting using antibodies indicated. (C) Mice with BCR-ABL-T315I–induced CML were treated with placebo, imatinib (100 mg/kg, twice a day by gavage), and IPI-504 (50 mg/kg, once every 2 days by gavage), respectively, for 8 days, beginning at day 8 after transplantation. At 6 hours after the last dose, protein lysates of leukemic cells from the spleen of the treated CML mice were analyzed by Western blotting using antibodies indicated. The black line indicates that the lanes that were not adjacent on the same original Western blotting gel were brought together to generate this figure. (D) Mice with BCR-ABL-T315I–induced B-ALL were treated with placebo, imatinib, and IPI-504, respectively, for 8 days, beginning at day 8 after transplantation. At 6 hours after the last dose, protein lysates of leukemic cells from the spleen of the treated mice were analyzed by Western blotting using antibodies indicated. The black line indicates that the lanes that were not adjacent on the same original Western blotting gel were brought together to generate this figure.

inhibitory effect on leukemia stem cells in CML, we first isolated bone marrow cells from mice with T315I-induced CML and cultured the cells in conditions that support survival and growth of HSCs.[28,29] During culture, the cells were treated with IPI-504 or imatinib (Figure 5). Six days after the treatment, we analyzed survival of GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$ cells, representing leukemia stem cells remaining in the culture. FACS analysis showed that compared with the untreated group, imatinib treatment did not lower the percentage and the number of leukemia stem cells, whereas IPI-504 treatment had a dramatic inhibitory effect on the stem cells (Figure 5A, $P < .001$). We next tested whether IPI-504 inhibits leukemia stem cells in CML mice. Mice with BCR-ABL-T315I–induced CML were treated with a placebo, imatinib, or IPI-504 for 6 days, and bone marrow cells were analyzed by FACS for GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$ cells. Consistent with the in vitro results, imatinib treatment did not lower the percentage and number of leukemia stem cells, compared with the untreated group, whereas IPI-504 treatment had a dramatic inhibitory effect on the stem cells (Figure 5B). To determine whether IPI-504 had an effect on normal HSCs in mice, WT mice were treated with IPI-504 or placebo for 2 weeks. Analysis of bone marrow from these mice showed that there was no change in levels of Lin$^-$c-Kit$^+$Sca-1$^+$ cells from any treatment group (Figure 5C), indicating that IPI-504 treatment did not inhibit survival of normal HSCs.

Inhibition of Hsp90 prevents emergence of the T315I-expressing clones over the WT clones

The effectiveness of IPI-504 in prolonging survival of mice with CML and B-ALL induced by the T315I mutant (Figures 2-3) suggests that inhibition of Hsp90 would preferentially prevent emergence of the T315I-expressing clones over the WT clones. To test this hypothesis, studies were performed in mice bearing both populations of leukemic cells. In the first study, bone marrow cells (BMCs) from Ly 5.1 and Ly5.2 C57BL/6 mice were transduced with BCR-ABL-T315I and BCR-ABL-WT, respectively. Equal numbers of donor BMCs were mixed and transplanted into recipient mice. Mice were treated with a placebo, imatinib, or IPI-504. During the treatment, FACS analysis was performed to assess the percentages of GFP$^+$Gr-1$^+$Ly5.1$^+$ (representing T315I-expressing cells) and GFP$^+$Gr-1$^+$Ly5.1$^-$ (representing WT-expressing cells) cells in peripheral blood of the CML mice (Figure 6A). In placebo-treated mice, the ratio between T315I- and WT-expressing cells remained unchanged, and in imatinib-treated mice, T315I-expressing cells became dominant. In contrast, with continuous treatment of IPI-504, T315I-expressing cells gradually decreased to a low level (Figure 6A). Mice treated with IPI-504 lived significantly longer than those treated with imatinib (Figure 6A). Consistent with previous data, these results indicate that inhibition of Hsp90 preferentially suppresses T315I-expressing leukemic clones over the WT-expressing clones. In the second study, BMCs from BALB/c mice were transduced with BCR-ABL-T315I and BCR-ABL-WT, respectively, and equal numbers of the transduced cells were mixed and transplanted into recipient mice. Mice were treated with a placebo, imatinib, IPI-504, or both agents (Figure 6B). Mice treated with the combination of IPI-504 and imatinib survived significantly longer than those treated with IPI-504 or imatinib alone. Results from these studies suggest that the combined use of IPI-504 and imatinib would be a viable strategy for preventing emergence of imatinib-resistant clones in the clinic.

Other imatinib-resistant BCR-ABL mutants are also sensitive to Hsp90 inhibition

Other resistance-conferring BCR-ABL kinase domain mutations have been observed in imatinib refractory CML patients, including E225K, M351T, and Y253F.[5,9] Consistent with the increased

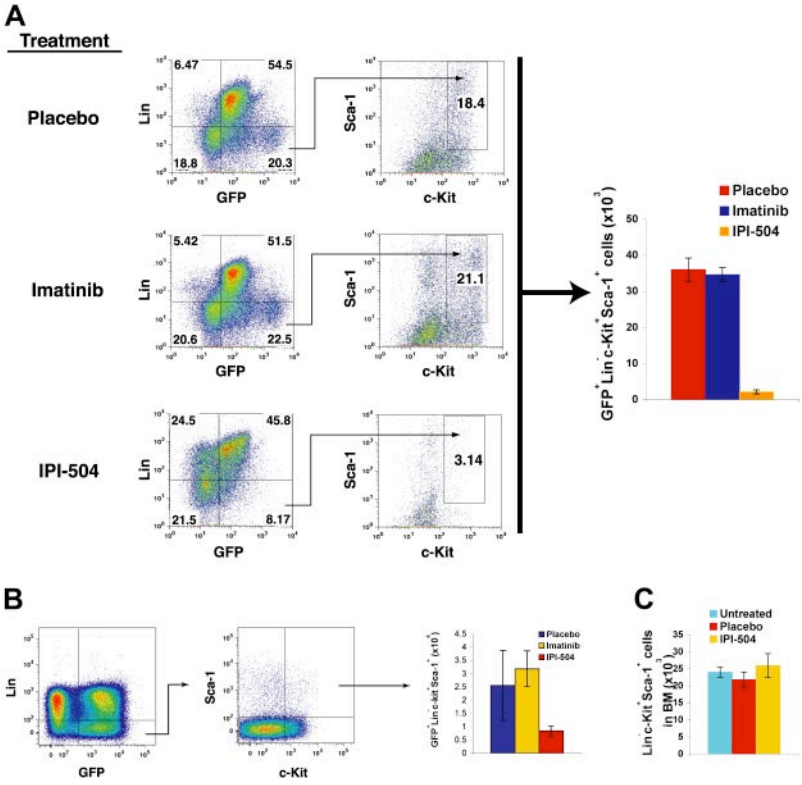

Figure 5. Targeting Hsp90 by IPI-504 inhibits survival of leukemia stem cells. (A) Bone marrow cells isolated from C57BL/6 (B6) mice with BCR-ABL-T315I–induced CML were cultured in vitro (5×10^6 cells/6 cm tissue culture plate) under the stem cell condition ("Materials and methods") in the presence or absence of IPI-504 (0.1 μM) or imatinib (2 μM) for 6 days (changing the stem cell medium containing placebo or IPI-504 at day 3) followed by FACS analysis of leukemia stem cells (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$). (B) Mice with BCR-ABL-T315I–induced CML were treated with a placebo (n = 5), imatinib (100 mg/kg, twice a day by gavage) (n = 5), and IPI-504 (50 mg/kg, once every 2 days by gavage) (n = 5), respectively, for 6 days beginning at day 8 after transplantation. Bone marrow cells were isolated from the treated CML mice, and leukemia stem cells were analyzed by FACS. The numbers of cells represent total leukemia stem cells in average from femur and tibia of each treated CML mouse. (C) IPI-504 had no inhibitory effect on survival of normal HSCs in mice. B6 mice were treated with a placebo (n = 5), imatinib (100 mg/kg, twice a day by gavage) (n = 5), and IPI-504 (50 mg/kg, once every 2 days by gavage) (n = 5), respectively, for 2 weeks. Bone marrow cells were isolated from the treated mice, and were analyzed by FACS.

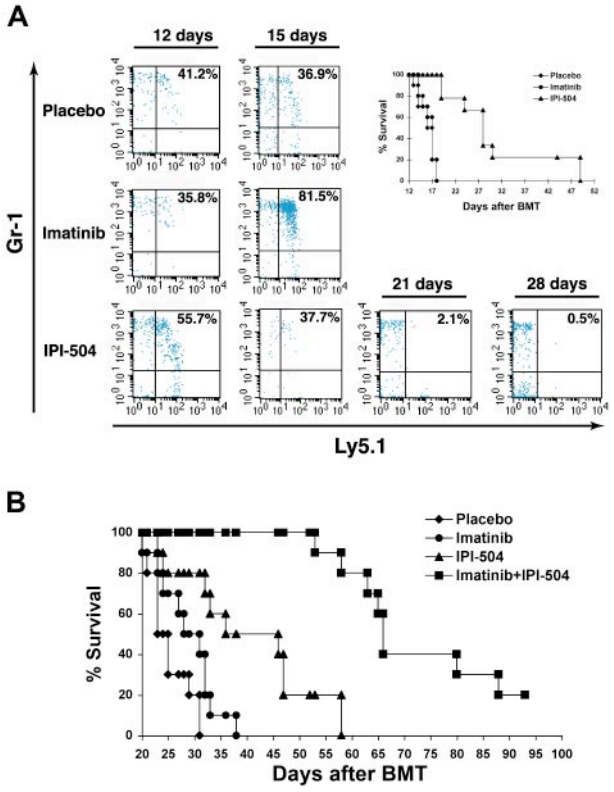

Figure 6. Inhibition of Hsp90 by IPI-504 preferentially reduces growth of myeloid leukemic cells harboring the BCR-ABL-T315I mutant. (A) Bone marrow cells from C57BL/6-Ly5.2 mice were transduced by BCR-ABL-WT, and bone marrow cells from C57BL/6-Ly5.1 mice were transduced by BCR-ABL-T315I. The transduced cells were 1:1 mixed, and 0.5×10^6 mixed cells were injected into each recipient mouse (C57BL/6-Ly5.2). The mice were treated with a placebo (n = 10), imatinib (100 mg/kg, twice a day) (n = 10), and IPI-504 (50 mg/kg, once every 2 days) (n = 10), respectively, beginning at 8 days after BMT. At days 12 and 15 after BMT, GFP^+ cells viable cells in peripheral blood of the mice were analyzed for $Gr\text{-}1^+Ly5.1^+$ cells that represented BCR-ABL-T315I–expressing myeloid cells. $Gr\text{-}1^+Ly5.1^-$ cells represented BCR-ABL-WT–expressing myeloid cells. Percentages of BCR-ABL-T315I–expressing myeloid cells in peripheral blood of IPI-504–treated CML mice were further analyzed at days 21 and 28 after BMT. The FACS results for one representative mouse from each treatment group were shown. IPI-504 but not imatinib significantly prolonged survival of the CML mice. (B) Simultaneous inhibition of Hsp90 and BCR-ABL kinase activity with IPI-504 and imatinib significantly prolongs survival of CML mice carrying both T315-expressing and WT-BCR-ABL leukemia cells. BALB/c mice were used to induce CML, and each treatment group had 10 mice.

dependency of BCR-ABL-T315I on Hsp90, IPI-504 also prolonged survival of mice with CML induced by these mutants (Figure 7).

Discussion

While the mechanism of primary resistance to imatinib and dasatinib therapy in CML patients is poorly understood, the mechanisms of secondary resistance have been very well characterized. Kinase domain mutations represent the predominant form of secondary resistance accounting for up to 90% of cases. Currently, no drugs have been effective in treating patients with CML and B-ALL harboring the BCR-ABL-T315I mutation. Recent clinical trials with dasatinib revealed that patients known to have the BCR-ABL-T315I mutation prior to therapy had no objective response to treatment.[16] Thus, as newer tyrosine kinase inhibitors (TKIs) that effectively block other resistant mutations become clinically available, the T315I mutation may become the predominant acquired resistance mutation. The challenge for development of an effective Ph^+ leukemia therapy is therefore to develop an alternative treatment strategy that does not rely solely on kinase domain inhibition but rather results in degradation of the offending BCR-ABL protein regardless of its mutational status. Herein, we demonstrate that direct inhibition of Hsp90 function with IPI-504 represents an alternative treatment strategy that results in degradation of the offending BCR-ABL protein regardless of its mutational status. Our findings suggest that inhibition of other targets that impact Hsp90 function might also be effective in murine models of Ph^+ leukemia. For example, histone deacetylatase (HDAC) inhibitors that induce acetylation and inhibition of Hsp90 might also be active.[41-44]

While imatinib induces complete hematologic and cytogenetic remission in the majority of newly diagnosed chronic-phase CML patients,[45] molecular remission is difficult to achieve in these patients. One study designed to look at newly diagnosed chronic-phase patients using the standard-dose imatinib (400 mg daily), with a 18-month follow-up showed that only 39% of patients obtained a major molecular response (greater than or equal to 3-log reduction of BCR-ABL mRNA), whereas even fewer patients, 4%, obtained a complete molecular response rate (negativity by reverse-transcription–polymerase chain reaction [RT-PCR]).[46] One prediction as to why the patients do not obtain a complete molecular response is perhaps due to imatinib not targeting the BCR-ABL–positive stem cells. This suggests that therapy with imatinib does not completely eradicate leukemic cells. It is likely that a small number of leukemic cells remain in imatinib-treated CML patients, and these cells may function as leukemia stem cells responsible for disease relapse. The inhibitory effects of IPI-504 on leukemia stem cells, while appearing to spare the normal hematopoietic stem cells, merits further investigation. Sole inhibition of BCR-ABL by imatinib has limited inhibitory effects on leukemic stem cells in mice.[40] Thus, a pathway distinct from BCR-ABL is likely involved in suppression of survival of leukemic stem cells by IPI-504. A plausible explanation is that BCR-ABL cooperates with a non–BCR-ABL signaling pathway that is driven by an unknown Hsp90 client protein to maintain survival of leukemic stem cells. IPI-504 is able to inhibit both pathways, as would be necessary to suppress leukemic stem cells. The putative pathway that is Hsp90 dependent might be less critical for normal hematopoietic stem cells. The putative non–BCR-ABL pathway in leukemic stem cells requires further study.

The inhibitory effects of IPI-504 on BCR-ABL-T315I–expressing cells indicate that Hsp90 may serve as an effective target for treating imatinib- and dasatinib-resistant CML patients, as well as patients with blast crisis or with Ph^+ ALL. The simultaneous use of IPI-504 and imatinib in chronic-phase CML patients might prevent the development of imatinib-resistant clones and inhibit growth of highly proliferative leukemic cells through inhibition of BCR-ABL kinase activity, thereby providing a rationale for combination strategy. Likewise, early use of IPI-504 to suppress initial B-ALL clones may help prevent the transition of

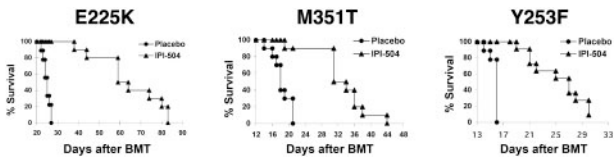

Figure 7. Other imatinib-resistant BCR-ABL mutants are also sensitive to Hsp90 inhibition. IPI-504 treatment prolonged survival of mice with CML induced by imatinib-resistant BCR-ABL-E225K (n = 10), -M351T (n = 10), or -Y253F (n = 10).

CML to advanced B-ALL caused by the BCR-ABL-T315I mutation. While IPI-504 was active in BCR-ABL–induced B-ALL, the activity was not as pronounced as in CML. Studies to evaluate the mechanism for this difference showed that Hsp70 was more strongly induced in myeloid cells compared with lymphoid cells. Hsp70 is reported to exert antiapoptotic effects in a variety of settings and cell types, including leukemia cells that are exposed to Hsp90 inhibitors.[37,38] In separate studies, inhibition of Hsp90 was shown to result in increased binding of BCR-ABL to Hsp70, thereby favoring proteasome-mediated degradation of BCR-ABL.[19,34-36] Thus, on one hand, Hsp70 induction could counter the effects of Hsp90 inhibition, while other studies suggest that Hsp70 could have a positive influence on the ability of Hsp90 inhibition to result in degradation of BCR-ABL. Elucidation of the mechanism of differential sensitivity to Hsp90 inhibition between myeloid and lymphoid leukemia will require more extensive studies, as variation in Hsp70 induction is not likely the cause.

In summary, IPI-504 represents a novel therapeutic approach whereby inhibition of Hsp90 in CML patients and Ph^+ ALL may significantly advance efforts to develop a cure for these diseases. The rationale underlying the use of IPI-504 for kinase inhibitor–resistant CML has implications for other cancers that display oncogene addiction to kinases that are Hsp90 client proteins. While resistant conferring kinase-domain mutations were originally described in CML, analogous mutations have been observed in lung cancer, gastrointestinal stromal tumor, and the hypereosinophilic syndrome with resistance to kinase inhibitor therapy.[47] IPI-504 is currently in clinical trials to evaluate its potential for treating cancer that has become resistant to therapy with tyrosine kinase inhibitors such as imatinib.

Acknowledgments

This work was supported by grants from the Department of Defense, and the National Cancer Institute (R01-CA114199) to S.L.

We thank Stephen B. Sampson for critical reading of the paper, Patricia Cherry for secretarial assistance, and Richard A. Van Etten for the BCR-ABL mutant constructs E225K, M351T, and Y253F.

Authorship

Contribution: C.P. performed experiments and analyzed the data; J.B. provided reagents and helped with the paper; Y.H., L.K., and A.G. helped with the experiments; D.G. provided reagents and helped with the paper; M.R. provided reagents and helped with the paper; R.P. provided reagents; S.L. designed and performed experiments, analyzed the data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Shaoguang Li, The Jackson Laboratory, 600 Main St, Bar Harbor, ME 04609; e-mail: shaoguang.li@jax.org.

References

1. Bartram CR, de Klein A, Hagemeijer A, et al. Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature. 1983;306:277-280.
2. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001;344:1031-1037.
3. Graham SM, Jorgensen HG, Allan E, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood. 2002;99:319-325.
4. Marley SB, Deininger MW, Davidson RJ, Goldman JM, Gordon MY. The tyrosine kinase inhibitor STI571, like interferon-alpha, preferentially reduces the capacity for amplification of granulocyte-macrophage progenitors from patients with chronic myeloid leukemia. Exp Hematol. 2000;28:551-557.
5. Gorre ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science. 2001;293:876-880.
6. Weisberg E, Griffin JD. Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines. Blood. 2000;95:3498-3505.
7. le Coutre P, Tassi E, Varella-Garcia M, et al. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. Blood. 2000;95:1758-1766.
8. Mahon FX, Deininger MW, Schultheis B, et al. Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. Blood. 2000;96:1070-1079.
9. Shah NP, Nicoll JM, Nagar B, et al. Multiple BCR-ABL kinase domain mutants confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crise chronic myeloid leukemia. Cancer Cell. 2002;2:117-125.
10. Branford S, Rudzki Z, Walsh S, et al. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood. 2002;99:3472-3475.
11. von Bubnoff N, Schneller F, Peschel C, Duyster J. BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study. Lancet. 2002;359:487-491.
12. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science. 2004;305:399-401.
13. O'Hare T, Pollock R, Stoffregen EP, et al. Inhibition of wild-type and mutant Bcr-Abl by AP23464, a potent ATP-based oncogenic protein kinase inhibitor: implications for CML. Blood. 2004;104:2532-2539.
14. Weisberg E, Manley PW, Breitenstein W, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell. 2005;7:129-141.
15. Druker BJ, Sawyers CL, Kantarjian H, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med. 2001;344:1038-1042.
16. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med. 2006;354:2531-2541.
17. Isaacs JS, Xu W, Neckers L. Heat shock protein 90 as a molecular target for cancer therapeutics. Cancer Cell. 2003;3:213-217.
18. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. Nat Rev Cancer. 2005;5:761-772.
19. An WG, Schulte TW, Neckers LM. The heat shock protein 90 antagonist geldanamycin alters chaperone association with p210bcr-abl and v-src proteins before their degradation by the proteasome. Cell Growth Differ. 2000;11:355-360.
20. Gorre ME, Ellwood-Yen K, Chiosis G, Rosen N, Sawyers CL. BCR-ABL point mutants isolated from patients with imatinib mesylate-resistant chronic myeloid leukemia remain sensitive to inhibitors of the BCR-ABL chaperone heat shock protein 90. Blood. 2002;100:3041-3044.
21. Kamal A, Thao L, Sensintaffar J, et al. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature. 2003;425:407-410.
22. Ge J, Normant E, Porter JR, et al. Design, synthesis, and biological evaluation of hydroquinone derivatives of 17-amino-17-demethoxygeldanamycin as potent, water-soluble inhibitors of Hsp90. J Med Chem. 2006;49:4606-4615.
23. Li S, Couvillon AD, Brasher BB, Van Etten RA. Tyrosine phosphorylation of Grb2 by Bcr/Abl and epidermal growth factor receptor: a novel regulatory mechanism for tyrosine kinase signaling. EMBO J. 2001;20:6793-6804.
24. Pear WS, Miller JP, Xu L, et al. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood. 1998;92:3780-3792.
25. Finer MH, Dull TJ, Qin L, Farson D, Roberts MR. kat: a high-efficiency retroviral transduction system for primary human T lymphocytes. Blood. 1994;83:43-50.
26. Li S, Ilaria RL Jr, Million RP, Daley GQ, Van Etten RA. The P190, P210, and p230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. J Exp Med. 1999;189:1399-1412.
27. Roumiantsev S, de Aos IE, Varticovski L, Ilaria RL, Van Etten RA. The src homology 2 domain of Bcr/Abl is required for efficient induction of chronic myeloid leukemia-like disease in mice but not for lymphoid leukemogenesis or activation of

phosphatidylinositol 3-kinase. Blood. 2001;97:4-13.

28. Zhang CC, Lodish HF. Murine hematopoietic stem cells change their surface phenotype during ex vivo expansion. Blood. 2005;105:4314-4320.
29. de Haan G, Weersing E, Dontje B, et al. In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1. Dev Cell. 2003;4:241-251.
30. Palombella VJ, Conner EM, Fuseler JW, et al. Role of the proteasome and NF-kappaB in streptococcal cell wall-induced polyarthritis. Proc Natl Acad Sci U S A. 1998;95:15671-15676.
31. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res. 1999;59:2615-2622.
32. Sawyers CL. Chronic myeloid leukemia. N Engl J Med. 1999;340:1330-1340.
33. Hu Y, Liu Y, Pelletier S, et al. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. Nat Genet. 2004;36:453-461.
34. Nimmanapalli R, O'Bryan E, Bhalla K. Geldanamycin and its analogue 17-allylamino-17-demethoxygeldanamycin lowers Bcr-Abl levels and induces apoptosis and differentiation of Bcr-Abl-positive human leukemic blasts. Cancer Res. 2001;61:1799-1804.
35. Nimmanapalli R, O'Bryan E, Huang M, et al. Molecular characterization and sensitivity of STI-571 (imatinib mesylate, Gleevec)-resistant, Bcr-Abl-positive, human acute leukemia cells to SRC kinase inhibitor PD180970 and 17-allylamino-17-demethoxygeldanamycin. Cancer Res. 2002;62:5761-5769.
36. Radujkovic A, Schad M, Topaly J, et al. Synergistic activity of imatinib and 17-AAG in imatinib-resistant CML cells overexpressing BCR-ABL: inhibition of P-glycoprotein function by 17-AAG. Leukemia. 2005;19:1198-1206.
37. Guo F, Sigua C, Bali P, et al. Mechanistic role of heat shock protein 70 in Bcr-Abl-mediated resistance to apoptosis in human acute leukemia cells. Blood. 2005;105:1246-1255.
38. Guo F, Rocha K, Bali P, et al. Abrogation of heat shock protein 70 induction as a strategy to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. Cancer Res. 2005;65:10536-10544.
39. Wolff NC, Ilaria RL Jr. Establishment of a murine model for therapy-treated chronic myelogenous leukemia using the tyrosine kinase inhibitor STI571. Blood. 2001;98:2808-2816.
40. Hu Y, Swerdlow S, Duffy TM, Weinmann R, Lee FY, Li S. Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph+ leukemia in mice. Proc Natl Acad Sci U S A. 2006;103:16870-16875.
41. Nimmanapalli R, Fuino L, Bali P, et al. Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory chronic myelogenous leukemia-blast crisis cells. Cancer Res. 2003;63:5126-5135.
42. Bali P, George P, Cohen P, et al. Superior activity of the combination of histone deacetylase inhibitor LAQ824 and the FLT-3 kinase inhibitor PKC412 against human acute myelogenous leukemia cells with mutant FLT-3. Clin Cancer Res. 2004;10:4991-4997.
43. Fiskus W, Pranpat M, Bali P, et al. Combined effects of novel tyrosine kinase inhibitor AMN107 and histone deacetylase inhibitor LBH589 against Bcr-Abl-expressing human leukemia cells. Blood. 2006;108:645-652.
44. George P, Bali P, Annavarapu S, et al. Combination of the histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC cells and AML cells with activating mutation of FLT-3. Blood. 2005;105:1768-1776.
45. Kantarjian H, Sawyers C, Hochhaus A, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med. 2002;346:645-652.
46. O'Brien SG, Deininger MW. Imatinib in patients with newly diagnosed chronic-phase chronic myeloid leukemia. Semin Hematol. 2003;40:26-30.
47. Baselga J. Targeting tyrosine kinases in cancer: the second wave. Science. 2006;312:1175-1178.

Stem Cells Review Series *J. Cell. Mol. Med. Vol 11, No 6, 2007 pp. 1251-1262*

Stem cell and kinase activity-independent pathway in resistance of leukaemia to BCR-ABL kinase inhibitors

Shaoguang Li [a, *], Dongguang Li [b]

[a] *The Jackson Laboratory, Bar Harbor, ME, USA*
[b] *School of Computer and Information Science, Edith Cowan University, Mount Lawley, WA, Australia*

Received: June 14, 2007; Accepted: August 23, 2007

Abstract

BCR-ABL tyrosine kinase inhibitors, such as imatinib (Gleevec) are highly effective in treating human Philadelphia chromosome-positive (Ph$^+$) chronic myeloid leukaemia (CML) in chronic phase but not in terminal acute phase; acquired drug resistance caused mainly by the development of BCR-ABL kinase domain mutations prevents cure of the leukaemia. In addition, imatinib is ineffective in treating Ph$^+$ B-cell acute lymphoblastic leukaemia (B-ALL) and CML blast crisis, even in the absence of the kinase domain mutations. This type of drug resistance that is unrelated to BCR-ABL kinase domain mutations is caused by the insensitivity of leukaemic stem cells to kinase inhibitors such as imatinib and dasatinib, and by activation of a newly-identified signalling pathway involving SRC kinases that are independent of BCR-ABL kinase activity for activation. This SRC pathway is essential for leukaemic cells to survive imatinib treatment and for CML transition to lymphoid blast crisis. Apart from BCR-ABL and SRC kinases, stem cell pathways must also be targeted for curative therapy of Ph$^+$ leukaemia.

Keywords: BCR-ABL • Ph$^+$ leukaemia • leukaemic stem cells • kinase inhibitors • SRC kinases • drug resistance

Introduction

The translocation between chromosomes 9 and 22 gives rise to the human Philadelphia chromosome, and results in formation of the chimeric BCR-ABL tyrosine kinase, a constitutively activated, oncogenic tyrosine kinase. Chronic myeloid leukaemia (CML) and B-cell acute lymphoblastic leukaemia (B-ALL) are both philadelphia chromosome-positive (Ph$^+$) leukaemias induced by the BCR-ABL oncogene.

*Correspondence to: Shaoguang LI,
The Jackson Laboratory,
600 Main Street, Bar Harbor, Maine, USA.
Tel.: 207-28 8-6734
Fax: 207-28 8-6978
E-mail: shaoguang.li@jax.org

doi: 10.1111/j.1582-4934.2007.00108.x

CML often initiates in a chronic phase and eventually progresses to a terminal blastic phase, in which either acute myeloid or acute B-lymphoid leukaemia develops. Some Ph^+ leukaemia patients, however, have B-ALL as their first clinical appearance. Because B-ALL is similar pathologically to acute B-lymphoid leukaemia in the blastic phase of CML, Ph^+ leukaemia may present as CML or B-ALL, and successful treatment of Ph + leukaemia requires management of both diseases induced by BCR-ABL. It is commonly believed that shutting down the kinase activity of BCR-ABL will completely inhibit its functions, leading to inactivation of its downstream signalling pathways. Therefore, current therapeutic efforts for Ph^+ leukaemia have focused on targeting BCR-ABL kinase activity using kinase inhibitors.

The BCR-ABL tyrosine kinase inhibitor imatinib mesylate (Gleevec) is the standard of care for Ph^+ leukaemia. Imatinib has been shown to induce a complete haematologic response in chronic phase CML patients [1]. However, imatinib has been unable to completely eliminate BCR-ABL-expressing leukaemic cells [2, 3], and patients frequently present with cellular and clinical drug resistance [4–10]. We and others have shown that imatinib prolongs survival of mice with BCR-ABL-induced CML [11, 12], but does not cure the disease [11]. Recently, three newly developed BCR-ABL kinase inhibitors, dasatinib [13], AP23464 [14] and AMN107 [15], have been shown to inhibit almost all imatinib-resistant BCR-ABL mutants in cell culture and animal studies; the exception is the BCR-ABL-T315I mutant, which is present in 15–20% of patients resistant to imatinib therapy. Besides its anti-BCR-ABL kinase activity, dasatinib is also a potent inhibitor of SRC family kinases, but the role of the anti-SRC activity of this compound in Ph^+ leukaemia therapy has not been studied [13]. For unknown reasons, imatinib is much less effective in treating CML blastic phase patients and patients with Ph^+ B-ALL [16, 17], which has not been shown to be related to the BCR-ABL kinase domain mutations, the most common type of imatinib-resistance. Because imatinib is a strong inhibitor of BCR-ABL kinase activity, the inability of imatinib to cure CML and B-ALL in mice [11] suggests that inactivation of BCR-ABL kinase activity alone is insufficient to control the disease.

We have previously shown that the three SRC family kinases LYN, HCK and FGR are activated by BCR-ABL in pre-B leukaemic cells and are required for the development of B-ALL [11]. We have also shown that imatinib does not cure mice with B-ALL, consistent with a study using human leukaemic cells, in which cells from patients resistant to imatinib expressed an activated form of LYN [18]. Recently, we have demonstrated that inhibition of SRC kinases and BCR-ABL by dasatinib is effective in controlling B-ALL in mice, but leukaemic stem cells in B-ALL or CML mice are insensitive to treatment with dasatinib or imatinib [19]. In this review, we focus on discussion of imatinib-resistant mechanisms that are not associated with mutations in BCR-ABL kinase domain because we believe that targeting these BCR-ABL kinase activity-independent pathways is critical to curative therapy of Ph^+ leukaemia.

Ph^+ leukaemia

The *BCR-ABL* oncogene is the cause of Ph^+ leukaemias. The *BCR* gene, on chromosome 22, breaks either at exon 1, exon 12/13 or exon 19 and fuses to the *c-ABL* gene on chromosome 9 to form, respectively, three types of BCR-ABL chimeric gene: P190, P210 or P230. Each of the three forms of the *BCR-ABL* oncogene is associated with a distinct type of human leukaemia. The P190 form is most often present in B-ALL but only rarely in CML [20], whereas P210 is mainly involved in CML and in some acute lymphoid [20] and myeloid leukaemias in CML blast crisis. P230 is found in a very mild form of CML [21]. Ph^+ B-ALL and lymphoid blast crisis of CML account for 20% of adults and 5% of children with acute B-lymphoid leukaemia. Among those patients with BCR-ABL-induced B-ALL, 50% of adults and 20% of children carry P210 form of *BCR-ABL* and the rest of the patients carry the P190 form [16, 20, 22].

Chronic phase CML responds to imatinib therapy [1]. The disease can progresses from chronic phase to accelerated phase or blast crisis, and the transition from chronic phase to blast crisis results in loss of imatinib response in Ph^+ leukaemia patients. Although the mechanism underlying the disease progression is not fully understood, additional genetic alterations are believed to play a role in this process. Mutations of tumour suppressor genes, such as the retinoblastoma gene (Rb), p16 and p53 appear to be associated with CML blast crisis patients [23–25]. However, it is still not known how BCR-ABL-expressing

J. Cell. Mol. Med. Vol 11, No 6, 2007

cells acquire these additional genetic lesions. An increase in genetic instability caused by BCR-ABL is one plausible mechanism, as BCR-ABL deregulates the functions of DNA repair-related genes according to several studies. For example, BCR-ABL down-reg-ulates expression of the DNA repair enzyme DNA-PKcs [26]. BCR-ABL may interact with the xeroderma pigmentosum group B protein, which could lead to the impairment of DNA repair function [27]. Expression of two other genes related to genetic stability, *BRCA-1* and *RAD51*, is also deregulated by BCR-ABL [28, 29]. BCR-ABL can also cause overexpression and increased activity of the error-prone polymerase β, leading to an increased mutagenesis [28]. A recent study showed that BCR-ABL associates with rad 3-related protein (ATR), which is involved in DNA repair, and inhibits activation of ATR following DNA damage, leading to alteration of cellular responses to DNA damage [30]. Although BCR-ABL is a primary driver for growth of leukaemic cells [31], it is believed that the concomitant effect of BCR-ABL on cell survival and DNA double strand break repair may lead to the acquisition of additional genetic lesions contributing to progression of CML [32]. These studies demonstrate that disruption of DNA repair mechanisms by BCR-ABL may lead to progression of chronic phase CML to more advance disease.

Leukaemic stem cells

A key characteristic of stem cells is the ability for self-renewal. Only long-term and short-term self-renewing haematopoietic stem cells (HSCs) have the ability to renew themselves, although other multi-potent progenitors in the haematopoietic system proliferate and differentiate to become more mature blood cells. [33]. Some multi-potent progenitors that are not normally self-renewing can aberrantly acquire self-renewing capacity during leukaemogenesis to become leukaemic stem cells. For example, granulocyte-macrophage progenitors have been identified as potential leukaemic stem cells for human CML myeloid blast crisis, and β-catenin that is involved in self-renewal of normal HSCs [34, 35] is also activated in granulocyte-macrophage progenitors [36], which appear to have acquired the potential for self-renewal through activation of β-catenin. It is still an open question whether cancer stem cells exist in all types of tumours; however, it is convincing that the cells capable of initiating human AML in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID) are exclusively $CD34^+CD38^-$ cells [37], which is characteristic of normal HSCs. This hints that leukaemic stem cells and normal HSCs may share mechanisms for regulation of self-renewal. This is supported by the studies using *Bim-1*-deficient mice, in which *Bim-1* is required for maintenance of self-renewal of normal HSCs [38, 39] and stem cells for AML induced co-operatively by the *Hoxa9* and *Meis1a* genes [38]. Evidence that Bim-1-related pathways play roles in the repopulating ability of the leukaemic stem cells is provided by the finding that $Bim1^{-/-}$ bone marrow cells from AML mice are incapable of re-producing the disease in secondary recipients [38]. However, failure to re-populate malignant diseases to secondary recipients does not exclude the possibility that the transferred cancer stem cells with self-renewal capability did not engraft due to complex mechanisms related to the donor–recipient interactions. This interaction between stem cells and their specific bone marrow microenvironment is critical for regulating the balance between self-renewal and differentiation of HSCs [40]. A new way of understanding physiopathology of human haematologic malignancies is to fully understand how leukaemic stem cells communicate with bone marrow microenvironment.

Identification of CML stem cells in mice

CML is believed to be a stem cell disease. BCR-ABL induces CML in mice [41–43], but mouse CML stem cells were not identified and characterized until last year. We first tested whether BCR-ABL-expressing HSCs function as the stem cells in mice. When C57BL/6 (B6) bone marrow cells transduced with BCR-ABL retrovirus were sorted into two separate populations ($Sca\text{-}1^-$ or $Sca\text{-}1^+$), only BCR-ABL-transduced $Sca\text{-}1^+$ cells transferred lethal CML to secondary B6 recipient mice [19], suggesting that early bone marrow progenitors contain CML stem cells. To narrow down the specific cell lineages that function as CML stem cells, HSCs ($Lin^-c\text{-}kit^+Sca\text{-}1^+$) were thought to be likely candidate population. Indeed, BCR-ABL-expressing HSCs ($GFP^+Lin\text{-}c\text{-}Kit^+Sca\text{-}1^+$)

isolated from bone marrow cells of primary CML mice induces CML in B6 recipient mice, indicating that BCR-ABL-expressing HSCs function as CML stem cells [19]. It is still to be tested whether other cell lineages serve as CML stem cells.

BCR-ABL kinase inhibitors prolong survival of CML mice, but do not completely eradicate CML stem cells

BCR-ABL kinase inhibitors are effective in treating CML, but are unlikely to provide a cure for the disease, as imatinib does not effectively kill BCR-ABL-expressing primitive human $CD34^+$ cells [2] and cure CML mice [11]. When a more potent BCR-ABL kinase inhibitor dasatinib [13] is used to treat CML mice, the mice lived significantly longer than those treated with imatinib, but eventually still died of this disease, indicating that, like imatinib [44], dasatinib may not eradicate leukaemic stem cells in CML mice because CML in mouse leukaemia model originates from multi-lineage repopulating cells [45]. When dasatinib is tested for killing BCR-ABL-expressing haematopoieticHSCs *in vivo*, BCR-ABL-expressing HSCs ($GFP^+CD34^-c\text{-}Kit^+$ Hoe^-) in the side population (SP) [46] of BM cells from the dasatinib-treated CML mice were not completely eradicated by dasatinib treatment [19]. This and other results suggest that neither dasatinib nor imatinib will cure CML and that targeting at least one additional component of leukaemic stem cells is required for curing the disease. The inability of dasatinib or possibly other kinase inhibitors to completely eradicate CML stem cells may be attributed to failure of the kinase inhibitors to access the stem cells. However, this is not the case, as dasatinib inhibited BCR-ABL phosporylation in the stem cells *in vivo* [19], indicating the drug entered and functioned in CML stem cells. The observed dasatinib-resistance of CML stem cells is not attributed to appearance of BCR-ABL-T315I (insensitive to dasatinib) clone in the mice, as sequencing analysis of genomic DNA from BM cells of these dasatinib-treated CML mice did not reveal the T315I mutation in the BCR-ABL kinase domain (data not shown). Wong *et al.* [47] show that c-kit is involved in CML development, however, the failure of imatinib and dasatinib to eradicate leukaemic stem cells is not related to c-kit function, as both drugs inhibit c-kit [48]. Together, these results demonstrate that inhibition of BCR-ABL kinase activity alone is insufficient to eradicate CML stem cells, leading to cure of the disease.

B-ALL stem cells and their sensitivity to kinase inhibitors

Although it is unclear what the stem cells for Ph^+ B-ALL are, Ph^+ B-ALL and CML could develop from a common stem cell, as chronic phase CML progresses to acute lymphoid leukaemia and Ph^+ B-ALL often coexists with CML [49]. This idea is supported by the observation that the same antiserum recognizes both B-ALL cells and cells from CML patients [50]. Furthermore, lymphoid and myeloid leukaemias induced by BCR-ABL originate from the same progenitor cells in mice [45]. On the other hand, Ph^+ B-ALL induced by P190 form of BCR-ABL is rarely present in CML [20], suggesting a possibility that early lymphoid progenitors become the stem cells for Ph^+ B-ALL. We have noticed that the majority of residual cells in dasatinib-treated B-ALL mice are BCR-ABL-expressing $B220^+/CD43^+$ and $B220^+/CD43^-$ pro-/pre-B cells, which may have acquired self-renewal capacity to function as B-ALL stem cells. We indeed observed that the $B220^+/CD19^+/GFP^+$ cells sorted from bone marrow of B-ALL mice transferred lymphoid leukaemia in lethally irradiated syngeneic mice [19], showing that BCR-ABL-expressing pro-B cells can function as B-ALL stem cells.

It is critical to test whether B-ALL is sensitive to treatment with BCR-ABL kinase inhibitors. Imatinib is weakly effective in treating B-ALL [11, 19]. Although dasatinib remarkably prolongs survival of B-ALL mice, there were still small amount of leukaemic cells remaining in peripheral blood of these mice, even after 3 months of treatment; when dasatinib treatment was stopped, B-ALL mice relapsed and died if not treated [19]. The relapsed B-ALL mice remained sensitive to dasatinib treatment, and the disease could be controlled by continuous use of this drug [19]. These results indicate that continuous dasatinib treatment could prevent the residual leukaemic cells

J. Cell. Mol. Med. Vol 11, No 6, 2007

(presumably B-ALL stem cells) from developing into fatal B-ALL, although this drug did not completely eradicate B-ALL stem cells.

Compared with dasatinib-treated CML mice that survived much longer than untreated mice, dasatinib-treated B-ALL mice survived continuously as long as the treatment continued [19]. The more superb therapeutic effect of dasatinib on B-ALL than on CML could be due to the activity of dasatinib against SRC kinases. As mentioned above, the pathways that regulate self-renewal of stem cells for haematologic malignancies involve the Bim-1 [38] and Wnt/β-catenin [36] pathways, and SRC kinases may be involved in regulation of the β-catenin pathway [51]. In addition, v-SRC has been shown to activate β-catenin-LEF/TCF (lymphoid enhancer factor/T-cell factor)-mediated transcription through the mitogen-activated protein kinase (MAPK) pathway [52]. A possible role of SRC kinases in self-renewal of Ph^+ leukaemia needs to be further studied.

SRC kinase may impact B-ALL stem cell functions through activation of the downstream signalling molecule β-catenin

Activation of β-catenin in the Wnt signalling pathway has been shown to play a role in self-renewal of HSCs and proliferation of tumour cells [33, 34, 53]. As mentioned above, this molecule is activated in granulocyte-macrophage progenitors of advanced phase CML patients and is involved in self-renewal ability of these cells *in vitro* [36]. The striking therapeutic effect of dasatinib, but not imatinib, on B-ALL [19] suggests that inhibition of SRC kinases not only played a key role in killing the highly proliferating leukaemic cells, but also may have an inhibitory effect on leukaemic stem cells, which would diminish the contribution of these stem cells to the disease. Therefore, we investigated whether SRC kinases are involved in activation of β-catenin in BCR-ABL-expressing cells. We observed that β-catenin is activated in the BCR-ABL-expressing mouse pre-B cell line (BaF/3) (Fig. 1A). To test whether activated SRC kinases directly activate β-catenin, we expressed v-SRC in BaF/3 cells. v-SRC activated β-catenin (Fig. 1B), suggesting that normal SRC kinases activated by BCR-ABL may activate β-catenin directly. To provide supporting evidence for this hypothesis, we first compared the levels of β-catenin expression in BCR-ABL-expressing pre-B leukaemic cells in the presence and absence of the SRC kinases Lyn, Hck and Fgr. We have previously shown that, while the lack of Lyn, Hck and Fgr causes a severe defect in the development of B-ALL, BCR-ABL-transduced *Lyn*$^{-/-}$*Hck*$^{-/-}$*Fgr*$^{-/-}$ bone marrow cells can grow under Whitlock-Witte culture conditions at high cell density [11]. Using these conditions to test the role of the three SRC kinases in β-catenin activation, we found that the level of β-catenin activation in *Lyn*$^{-/-}$*Hck*$^{-/-}$*Fgr*$^{-/-}$ leukaemic cells was lower than that in wild-type leukaemic cells (Fig. 1C). To test whether inhibition of SRC kinases by dasatinib down-regulates β-catenin activation, we treated BCR-ABL-T315I-expressing pre-B leukaemic cells with dasatinib. β-catenin activation was inhibited by dasatinib at 100 nM, and this was associated with complete inhibition of SRC activation (Fig. 1D). These results link SRC kinases to β-catenin activation, implying that Src kinase may impact B-ALL stem cell functions through activation of the downstream signalling molecule β-catenin.

Activation of SRC kinases by BCR-ABL is independent of its kinase activity

BCR-ABL activates SRC kinases in myeloid cells, and SRC kinase inhibitors impair cellular transformation by BCR-ABL in cultured cells [54]. Stimulatory role of SRC kinases in transformation of lymphoid cells by BCR-ABL is demonstrated clearly using mice deficient for three SRC kinases LYN, HCK and FGR, and these three SRC kinases are shown to play a specific role in the induction of B-ALL not CML [11]. This lineage-specific function of SRC kinases indicates that SRC kinases are good therapeutic targets for B-ALL. The role of Lyn in BCR-ABL-induced lymphoid leukaemia is also supported by a study using lymphoid blastic cells from CML patients [55]. Lyn overexpression or inhibition of Lyn by the Src kinase inhibitor PP2 leads to an increase or decrease of Bcl-2 expression in LAMA84 and K562 cells [56], suggesting Bcl-2 may be a downstream signalling molecule of Lyn in BCR-ABL-positive cells. Because BCR-ABL activates SRC kinases, it is reasonable to think that inhibition of BCR-ABL kinase activity by imatinib would shut down SRC kinases

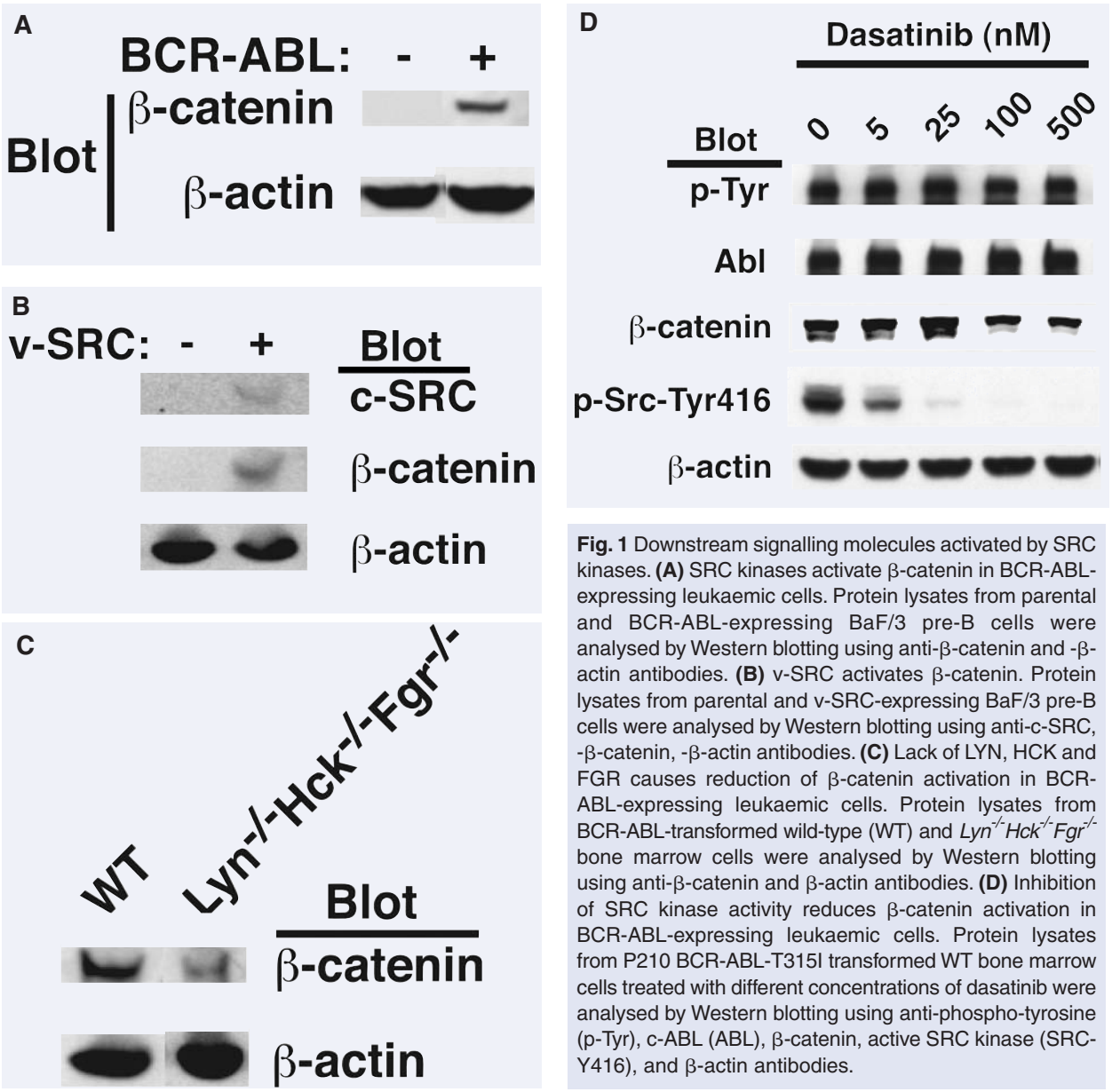

Fig. 1 Downstream signalling molecules activated by SRC kinases. **(A)** SRC kinases activate β-catenin in BCR-ABL-expressing leukaemic cells. Protein lysates from parental and BCR-ABL-expressing BaF/3 pre-B cells were analysed by Western blotting using anti-β-catenin and -β-actin antibodies. **(B)** v-SRC activates β-catenin. Protein lysates from parental and v-SRC-expressing BaF/3 pre-B cells were analysed by Western blotting using anti-c-SRC, -β-catenin, -β-actin antibodies. **(C)** Lack of LYN, HCK and FGR causes reduction of β-catenin activation in BCR-ABL-expressing leukaemic cells. Protein lysates from BCR-ABL-transformed wild-type (WT) and *Lyn*$^{-/-}$*Hck*$^{-/-}$*Fgr*$^{-/-}$ bone marrow cells were analysed by Western blotting using anti-β-catenin and β-actin antibodies. **(D)** Inhibition of SRC kinase activity reduces β-catenin activation in BCR-ABL-expressing leukaemic cells. Protein lysates from P210 BCR-ABL-T315I transformed WT bone marrow cells treated with different concentrations of dasatinib were analysed by Western blotting using anti-phospho-tyrosine (p-Tyr), c-ABL (ABL), β-catenin, active SRC kinase (SRC-Y416), and β-actin antibodies.

that are downstream of BCR-ABL. The striking finding is that in B-lymphoid cells imatinib markedly inhibited BCR-ABL kinase activity but did not result in a decrease in SRC activation, indicating that while imatinib was very effective in inhibiting BCR-ABL phosphorylation, it was unable to affect BCR-ABL-stimulated phosphorylation of SRC kinases [19]. These observations indicate that activation of SRC kinases by BCR-ABL is independent upon its kinase activity, suggesting the necessity of targeting both BCR-ABL and SRC kinases in treating B-ALL. The inability of imatinib to inactivate SRC kinases may explain the relatively poor activity of this drug against Ph$^+$ B-ALL and acute lymphoid leukaemia.

Role of SRC kinases in the development of B-ALL

As mentioned above, the critical role of SRC kinases in the development of BCR-ABL-induced is first

J. Cell. Mol. Med. Vol 11, No 6, 2007

demonstrated using the SRC-deficient mice [11]. Are SRC kinases good therapeutic targets for B-ALL? This question was answered by treating B-ALL mice with the dual SRC/ABL inhibitor dasatinib. The P210 form of BCR-ABL-T315I mutant is resistant to inhibition by both imatinib [4, 54, 57] and dasatinib [13]. Treatment of mice with B-ALL induced by BCR-ABL-T315I with imatinib or dasatinib showed that imatinib had no therapeutic effect, whereas dasatinib significantly prolonged survival of the mice [19], indicating that targeting SRC kinases alone prolongs survival of B-ALL mice. However, targeting SRC kinases alone did not cure the disease, which may be due to the incomplete inhibition of SRC kinase activity *in vivo*. Testing a stronger SRC inhibitor would help to reveal the full potential of SRC kinases as therapeutic targets for B-ALL induced by BCR-ABL.

The role of SRC kinases in B-ALL development is further supported by comparing growth potential of BCR-ABL-transduced wild-type (WT) and *Lyn*$^{-/-}$*Hck*$^{-/-}$*Fgr*$^{-/-}$ bone marrow cells [19]. Levels of BCR-ABL-expressing B220^{+} B-lymphoid leukaemic cells were significantly lower in mice receiving BCR-ABL transduced *Lyn*$^{-/-}$*Hck*$^{-/-}$*Fgr*$^{-/-}$ bone marrow cells than in those receiving BCR-ABL transduced WT bone marrow cells. Strikingly, B-lymphoid leukaemic cells in some mice receiving the transduced *Lyn*$^{-/-}$*Hck*$^{-/-}$*Fgr*$^{-/-}$ bone marrow cells almost disappeared 5 weeks following B-ALL induction, demonstrating more definitively a critical role of SRC kinases in B-ALL development.

Inhibition BCR-ABL kinase activity and SRC kinase leads to long-term survival of B-ALL mice

Imatinib only has a weak therapeutic effect on B–ALL [19], and this is likely due to the fact that SRC kinases are still active when BCR-ABL phosphorylation is inhibited by imatinib [19]. If so, shutting down simultaneously both SRC kinases and BCR-ABL kinase activity with dasatinib should provide much more dramatic therapeutic effect on B-ALL. Indeed, dasatinib maintained long-term survival of the mice with B-ALL induced by P190 or P210 form of BCR-ABL. The weak therapeutic effect of imatinib is not attributed to an inability to inhibit BCR-ABL kinase activity *in vivo*, as imatinib significantly inhibited BCR-ABL phosphorylation, to similar extend compared to dasatinib, in B-lymphoid leukaemic cells from the treated B-ALL mice. These results support the critical role of SRC kinases in B-ALL development.

Role of SRC kinases in progression of CML to lymphoid blast crisis

Additional genetic alterations, such as mutations in the tumour suppressor genes INK4^{a}, pRB and p53 are associated with the transition from CML chronic phase to acute (blastic) phase [23–25]. A recent study showed that Arf gene loss enhances oncogenicity of and limits imatinib response to BCR-ABL-induced B-ALL in mice [58]. Chronic phase CML responds to imatinib treatment, but imatinib becomes much less effective after the disease advances to blastic phase. In mice, serial transplantation of CML bone marrow cells to recipient mice leads to developing acute lymphoid leukaemia [59]. When mice were transplanted with BCR-ABL transduced bone marrow cells from either WT or *Lyn*$^{-/-}$*Hck*$^{-/-}$*Fgr*$^{-/-}$ mice to induce CML, followed by subsequently transferring bone marrow CML cells into lethally irradiated syngeneic recipient mice, mice receiving WT CML bone marrow cells developed B-ALL, whereas none of the mice receiving *Lyn*$^{-/-}$*Hck*$^{-/-}$*Fgr*$^{-/-}$ CML BM cells developed this disease. This result indicates that CML transition to lymphoid blast phase requires SRC kinases. Inhibition of SRC kinases by dasatinib may reflect therapeutic effect of this drug on B-ALL patients [60, 61]. It is important to mention that targeting SRC kinases are less effective in treating B-ALL when tumour suppressor gene function is defective, as dasatinib-treated recipients of BCR-AB-transduced bone marrow cells from p53-deficient mice survived longer than those treated with imatinib, but eventually died [19]. These results suggest that loss of tumour suppressor function impedes effectiveness of dasatinib or likely other kinase inhibitors in treating BCR-ABL-induced B-ALL, and identification of mutations in these tumour suppressor genes in patients is useful in guiding drug therapy of Ph^{+} leukaemia.

Comments and conclusions

It is still an open question whether BCR-ABL kinase inhibitors cure Ph^{+} leukaemia. Studies in mice show

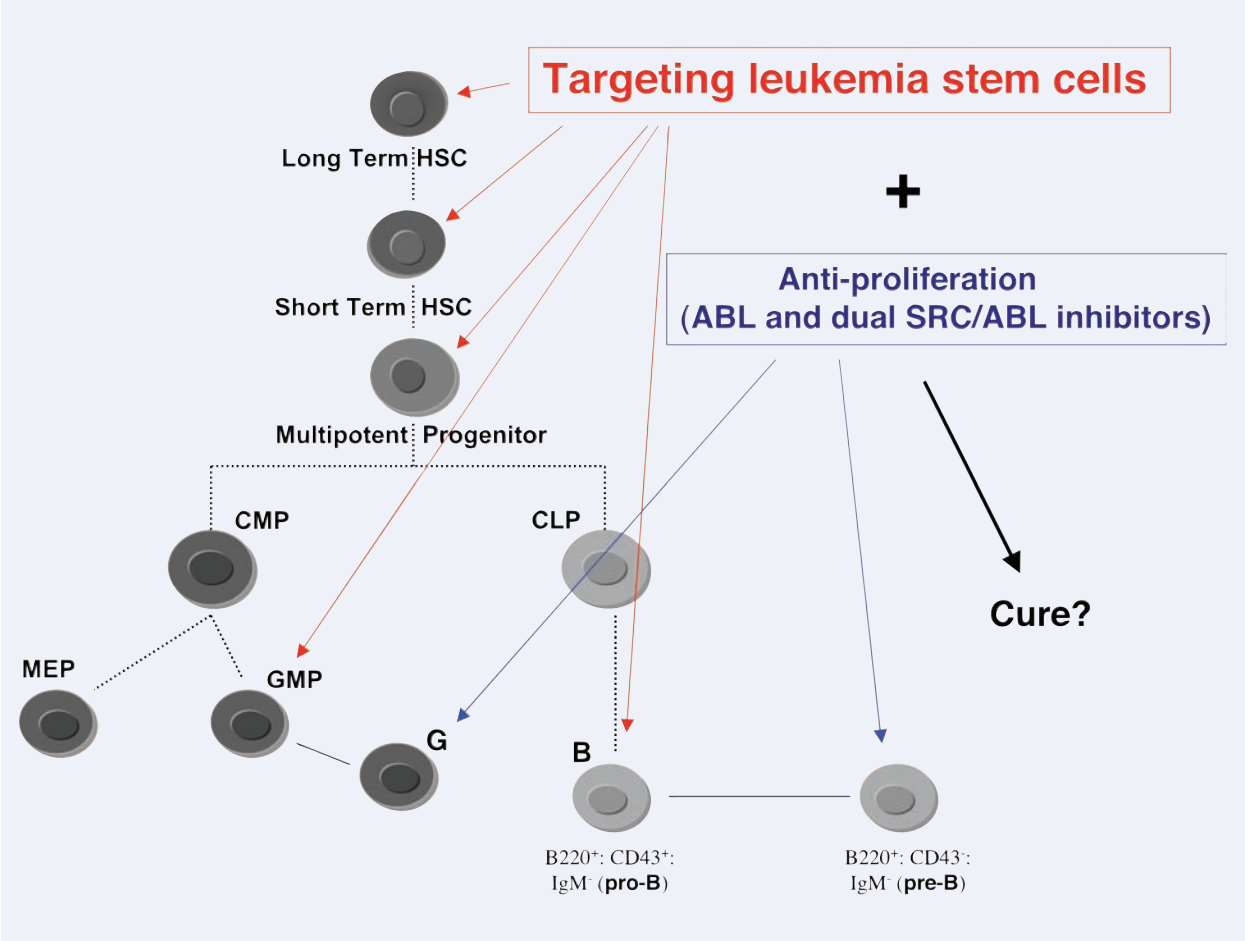

Fig. 2 Simultaneous targeting of both leukaemic stem cells and highly proliferative leukaemic cells may lead to cure of Ph^+ leukaemia. Treatment with BCR-ABL kinase inhibitors does not cure CML and B-ALL induced by BCR-ABL in mice, this is likely due to the inability of these inhibitors to kill leukaemic stem cells. Therefore, combination of anti-stem cell agents and BCR-ABL kinase inhibitors would be a promising therapeutic strategy for Ph^+ leukaemia.

that sole inhibition of BCR-ABL kinase activity by imatinib has an effect in treating Ph^+ B-ALL and CML but is not sufficient to achieve complete control of the diseases. One of the reasons is because inhibition of BCR-ABL kinase activity by imatinib does not inactivate some BCR-ABL activated signalling pathways such as SRC kinases, which are essential to B-ALL development. Sustained activation of these pathways would allow leukaemic cells to survive treatment with drugs that inhibit only BCR-ABL kinase activity, allowing accumulation of BCR-ABL mutations associated with drug resistance. Thus, simultaneous targeting of these BCR-ABL kinase activity-independent pathways and BCR-ABL kinase activity would provide a significantly improved therapeutic strategy for Ph^+ leukaemia. This strategy is against the idea that complete and sole inhibition of BCR-ABL kinase activity would completely inhibit BCR-ABL functions. On the other hand, BCR-ABL-activated SRC kinases alone may not be efficient in transformation of B-lymphoid cells, however, they are sufficient to maintain survival and stimulate proliferation of the leukaemic cells under treatment of BCR-ABL kinase inhibitors. Although the next generation of BCR-ABL kinase inhibitors aims at increasing drug potency or overriding imatinib resistance, BCR-ABL kinase activity-independent pathways must be targeted to achieve a durable therapeutic effect in patients with Ph^+ acute lymphoid leukaemia. Effectiveness of dasatinib in preventing or delaying transition of CML chronic

J. Cell. Mol. Med. Vol 11, No 6, 2007

phase to acute lymphoid leukaemia and in treating acute lymphoid leukaemia with compromised tumour suppressor function provides a rationale for the early and continuous use of dasatinib in chronic phase CML patients. Comparing to patients with compromised tumour suppressor functions, the early use of dasatinib would be more effective in preventing transition of patients with chronic phase CML to lymphoid blast crisis and for management of patients with advanced lymphoid leukaemia. This idea is indirectly supported by the clinical observation that dasatinib is effective in treating Ph^+ B-ALL patients [60]. Our thoughts are that, if the BCR-ABL-T315I mutation that does not respond to dasatinib treatment is absent from the leukaemic cell population, dasatinib treatment may lead to long-term remission of B-ALL.

Most challenging issue in therapy of Ph^+ leukaemia deals with leukaemic stem cells. Although dasatinib could help achieve long-term control of B-ALL in mice, curative drug therapy of this disease would require targeting quiescent leukaemic stem cells [62] in addition to BCR-ABL kinase activity and SRC-dependent pathways. Identification of CML and B-ALL stem cells in mice [19] is significant, as it provides a model system for studying the biology of leukaemic stem cells. Identification of pro-B leukaemic cells as stem cells for B-ALL is important, as it indicates that pro-B progenitors could acquire self-renewal capacity to become the major source of highly proliferating B-lymphoid leukaemic cells in B-ALL mice. Therefore, complete inhibition of growth of this leukaemic population could achieve long-term survival of B-ALL mice. This also promotes the effort in testing whether other progenitor lineages could also acquire stem-like properties. It will be important to assess whether the stem cells identified in leukaemic mice can be similarly found in Ph^+ leukaemia patients. Finally, insensitivity of leukaemic stem cells in mice to inhibition by both imatinib and dasatinib [19] prompts us to identify unknown pathways in leukaemic stem cells for developing curative therapies for Ph^+ leukaemia (Fig. 2). It is important to mention that in human CML patients the ineffectiveness of kinase inhibitors to completely eradicate leukaemic cells could also be due to the pre-existing BCR-ABL kinase domain mutations, as shown by the elegant work from Ottmann's group [63]. It will be critical to investigate whether these mutations exist in leukaemic stem cells of CML patients before they are treated with kinase inhibitors. On the other hand, there are other kinase inhibitors that inhibit multiple kinases in cancer cells. For example, the Aurora kinase VX-680 (MK-0457) suppresses tumour cell growth and also inhibits BCR-ABL kinase including imatinib-resistant mutant BCR-ABL [64–69]. It is worth testing whether this kind of inhibitors would have an inhibitory effect on leukaemic stem cells.

Acknowledgements

We thank Patricia Cherry for the secretarial assistance. Supported by the grants from the Department of Defense and the National Cancer Institute (CA114199) to S.L.

References

1. **Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL.** Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med.* 2001; 344: 1031–7.
2. **Graham SM, Jorgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, Holyoake TL.** Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 *in vitro*. *Blood.* 2002; 99: 319–25.
3. **Marley SB, Deininger MW, Davidson RJ, Goldman JM, Gordon MY.** The tyrosine kinase inhibitor STI571, like interferon-alpha, preferentially reduces the capacity for amplification of granulocyte-macrophage progenitors from patients with chronic myeloid leukemia. *Exp Hematol.* 2000; 28: 551–7.
4. **Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL.** Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science.* 2001; 293: 876–80.
5. **Weisberg E, Griffin JD.** Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines. *Blood.* 2000; 95: 3498–505.
6. **le Coutre P, Tassi E, Varella-Garcia M, Barni R, Mologni L, Cabrita G, Marchesi E, Supino R, Gambacorti-Passerini C.** Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood.* 2000; 95: 1758–66.
7. **Mahon FX, Deininger MW, Schultheis B, Chabrol J, Reiffers J, Goldman JM, Melo JV.** Selection and

characterization of *BCR-ABL* positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. *Blood.* 2000; 96: 1070–9.

8. **Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, Sawyers CL.** Multiple *BCR-ABL* kinase domain mutants confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crise chronic myeloid leukemia. *Cancer Cell.* 2002; 2: 117–25.
9. **Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, Herrmann R, Lynch KP, Hughes TP.** High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. *Blood.* 2002; 99: 3472–5.
10. **von Bubnoff N, Schneller F, Peschel C, Duyster J.** BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study. *Lancet.* 2002; 359: 487–91.
11. **Hu Y, Liu Y, Pelletier S, Buchdunger E, Warmuth M, Fabbro D, Hallek M, Van Etten RA, Li S.** Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. *Nat Genet.* 2004; 36: 453–61.
12. **Wolff NC, Ilaria RL, Jr.** Establishment of a murine model for therapy-treated chronic myelogenous leukemia using the tyrosine kinase inhibitor STI571. *Blood.* 2001; 98: 2808–16.
13. **Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL.** Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science.* 2004; 305: 399–401.
14. **O'Hare T, Pollock R, Stoffregen EP, Keats JA, Abdullah OM, Moseson EM, Rivera VM, Tang H, Metcalf CA 3rd, Bohacek RS, Wang Y, Sundaramoorthi R, Shakespeare WC, Dalgarno D, Clackson T, Sawyer TK, Deininger MW, Druker BJ.** Inhibition of wild-type and mutant Bcr-Abl by AP23464, a potent ATP-based oncogenic protein kinase inhibitor: implications for CML. *Blood.* 2004; 104: 2532–9.
15. **Weisberg E, Manley PW, Breitenstein W, Bruggen J, Cowan-Jacob SW, Ray A, Huntly B, Fabbro D, Fendrich G, Hall-Meyers E, Kung AL, Mestan J, Daley GQ, Callahan L, Catley L, Cavazza C, Mohammed A, Neuberg D, Wright RD, Gilliland DG, Griffin JD.** Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell.* 2005; 7: 129–41.
16. **Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M.** Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med.* 2001; 344: 1038–42.
17. **Talpaz M, Sawyers CL, Kantarjain H, Resta D, Fernandes Rees S, Ford J, Bruker BJ.** Activity of an ABL specific tyrosine kinase inhibitor in patients with BCR/ABL positive acute leukemias, including chronic myelogenous leukemia in blast crisis. *Oncologist.* 2000; 5: 282–3.
18. **Donato NJ, Wu JY, Stapley J, Gallick G, Lin H, Arlinghaus R, Talpaz M.** BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. *Blood.* 2003; 101: 690–8.
19. **Hu Y, Swerdlow S, Duffy TM, Weinmann R, Lee FY, Li S.** Targeting multiple kinase pathways in leukemic prognitors and stem cells is essential for improved treatment of Ph^+ leukemia in mice. *Proc Natl Acad Sci USA.* 2006; 103: 16870–75.
20. **Deininger MW, Goldman JM, Melo JV.** The molecular biology of chronic myeloid leukemia. *Blood.* 2000; 96: 3343–56.
21. **Pane F, Frigeri F, Sindona M, Luciano L, Ferrara F, Cimino R, Meloni G, Saglio G, Salvatore F, Rotoli B.** Neutrophilic-chronic myeloid leukemia: a distinct disease with a specific molecular marker (*BCR/ABL* with C3/A2 junction). *Blood.* 1996; 88: 2410–4.
22. **Sawyers CL.** Chronic myeloid leukemia. *N Engl J Med.* 1999; 340: 1330–40.
23. **Towatari M, Adachi K, Kato H, Saito H.** Absence of the human retinoblastoma gene product in the megakaryoblastic crisis of chronic myelogenous leukemia. *Blood.* 1991; 78: 2178–81.
24. **Sill H, Goldman JM, Cross NC.** Homozygous deletions of the p16 tumor-suppressor gene are associated with lymphoid transformation of chronic myeloid leukemia. *Blood.* 1995; 85: 2013–6.
25. **Feinstein E, Cimino G, Gale RP, Alimena G, Berthier R, Kishi K, Goldman J, Zaccaria A, Berrebi A, Canaani E.** p53 in chronic myelogenous leukemia in acute phase. *Proc Natl Acad Sci USA.* 1991; 88: 6293–7.
26. **Deutsch E, Dugray A, AbdulKarim B, Marangoni E, Maggiorella L, Vaganay S, M'Kacher R, Rasy SD, Eschwege F, Vainchenker W, Turhan AG, Bourhis J.** BCR-ABL down-regulates the DNA repair protein DNA-PKcs. *Blood.* 2001; 97: 2084–90.
27. **Takeda N, Shibuya M, Maru Y.** The BCR-ABL oncoprotein potentially interacts with the xeroderma pigmentosum group B protein. *Proc Natl Acad Sci USA.* 1999; 96: 203–7.
28. **Canitrot Y, Lautier D, Laurent G, Frechet M, Ahmed A, Turhan AG, Salles B, Cazaux C, Hoffmann JS.** Mutator phenotype of BCR–ABL transfected Ba/F3 cell lines and its association with enhanced expression of DNA polymerase beta. *Oncogene.* 1999; 18: 2676–80.

29. **Slupianek A, Schmutte C, Tombline G, Nieborowska-Skorska M, Hoser G, Nowicki MO, Pierce AJ, Fishel R, Skorski T.** BCR/ABL regulates mammalian RecA homologs, resulting in drug resistance. *Mol Cell.* 2001; 8: 795–806.
30. **Dierov J, Dierova R, Carroll M.** BCR/ABL translocates to the nucleus and disrupts an ATR-dependent intra-S phase checkpoint. *Cancer Cell.* 2004; 5: 275–85.
31. **Huettner CS, Zhang P, Van Etten RA, Tenen DG.** Reversibility of acute B-cell leukaemia induced by BCR-ABL1. *Nat Genet.* 2000; 24: 57–60.
32. **Calabretta B, Perrotti D.** The biology of CML blast crisis. *Blood.* 2004; 103: 4010–22.
33. **Reya T, Morrison SJ, Clarke MF, Weissman IL.** Stem cells, cancer, and cancer stem cells. *Nature.* 2001; 414: 105–11.
34. **Reya T, Duncan AW, Ailles L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL.** A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature.* 2003; 423: 409–14.
35. **Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR, 3rd, Nusse R.** Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature.* 2003; 423: 448–52.
36. **Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, Gotlib J, Li K, Manz MG, Keating A, Sawyers CL, Weissman IL.** Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med.* 2004; 351: 657–67.
37. **Bonnet D, Dick JE.** Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1997; 3: 730–7.
38. **Lessard J, Sauvageau G.** Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature.* 2003; 423: 255–60.
39. **Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ, Clarke MF.** Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature.* 2003; 423: 302–5.
40. **Wilson A, Murphy MJ, Oskarsson T, Kaloulis K, Bettess MD, Oser GM, Pasche AC, Knabenhans C, Macdonald HR, Trumpp A.** c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes Dev.* 2004; 18: 2747–63.
41. **Daley GQ, Van Etten RA, Baltimore D.** Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science.* 1990; 247: 824–30.
42. **Elefanty AG, Hariharan IK, Cory S.** bcr-abl, the hallmark of chronic myeloid leukaemia in man, induces multiple haemopoietic neoplasms in mice. *Embo J.* 1990; 9: 1069–78.
43. **Kelliher MA, McLaughlin J, Witte ON, Rosenberg N.** Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and BCR/ABL. *Proc Natl Acad Sci USA.* 1990; 87: 6649–53.
44. **Holtz MS, Slovak ML, Zhang F, Sawyers CL, Forman SJ, Bhatia R.** Imatinib mesylate (ST1571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation. *Blood.* 2002; 99: 3792–800.
45. **Li S, Ilaria RL, Jr., Million RP, Daley GQ, Van Etten RA.** The P190, P210, and p230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. *J Exp Med.* 1999; 189: 1399–412.
46. **Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC.** Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med.* 1996; 183: 1797–806.
47. **Wong S, McLaughlin J, Cheng D, Zhang C, Shokat KM, Witte ON.** Sole BCR-ABL inhibition is insufficient to eliminate all myeloproliferative disorder cell populations. *Proc Natl Acad Sci USA.* 2004; 101: 17456–61.
48. **Heinrich MC, Blanke CD, Druker BJ, Corless CL.** Inhibition of KIT tyrosine kinase activity: a novel molecular approach to the treatment of KIT-positive malignancies. *J Clin Oncol.* 2002; 20: 1692–703.
49. **Deininger M.** Src kinases in Ph^+ lymphoblastic leukemia. *Nat Genet.* 2004; 36: 440–1.
50. **Janossy G, Roberts M, Greaves MF.** Target cell in chronic myeloid leukaemia and its relationship to acute lymphoid leukaemia. *Lancet.* 1976; 2: 1058–61.
51. **Schroeder JA, Adriance MC, Thompson MC, Camenisch TD, Gendler SJ.** MUC1 alters beta-catenin- dependent tumor formation and promotes cellular invasion. *Oncogene.* 2003; 22: 1324–32.
52. **Haraguchi K, Nishida A, Ishidate T, Akiyama T.** Activation of beta-catenin-TCF-mediated transcription by non-receptor tyrosine kinase v-Src. *Biochem Biophys Res Commun.* 2004; 313: 841–4.
53. **Reya T.** Regulation of hematopoietic stem cell self-renewal. *Recent Prog Horm Res.* 2003; 58: 283–95.
54. **Warmuth M, Simon N, Mitina O, Mathes R, Fabbro D, Manley PW, Buchdunger E, Forster K, Moarefi I, Hallek M.** Dual-specific Src and Abl kinase inhibitors, PP1 and CGP76030, inhibit growth and survival of cells expressing imatinib mesylate-resistant Bcr-Abl kinases. *Blood.* 2003; 101: 664–72.
55. **Ptasznik A, Nakata Y, Kalota A, Emerson SG, Gewirtz AM.** Short interfering RNA (siRNA) targeting the Lyn kinase induces apoptosis in primary, and drug-resistant, BCR-ABL1(+) leukemia cells. *Nat Med.* 2004; 10: 1187–9.
56. **Dai Y, Rahmani M, Corey SJ, Dent P, Grant S.** A Bcr/Abl-independent, Lyn-dependent form of imatinib

mesylate (STI-571) resistance is associated with altered expression of Bcl-2. *J Biol Chem.* 2004; 279: 34227–39.

57. **Roumiantsev S, Shah NP, Gorre ME, Nicoll J, Brasher BB, Sawyers CL, Van Etten RA.** Clinical resistance to the kinase inhibitor STI-571 in chronic myeloid leukemia by mutation of Tyr-253 in the Abl kinase domain P-loop. *Proc Natl Acad Sci USA.* 2002; 99: 10700–5.
58. **Williams RT, Roussel MF, Sherr CJ.** Arf gene loss enhances oncogenicity and limits imatinib response in mouse models of Bcr-Abl-induced acute lymphoblastic leukemia. *Proc Natl Acad Sci USA.* 2006; 103: 6688–93.
59. **Pear WS, Miller JP, Xu L, Pui JC, Soffer B, Quackenbush RC, Pendergast AM, Bronson R, Aster JC, Scott ML, Baltimore D.** Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood.* 1998; 92: 3780–92.
60. **Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, Paquette R, Cortes J, O'Brien S, Nicaise C, Bleickardt E, Blackwood-Chirchir MA, Iyer V, Chen TT, Huang F, Decillis AP, Sawyers CL.** Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med.* 2006; 354: 2531–41.
61. **Quintas-Cardama A, Kantarjian H, Jones D, Nicaise C, O'Brien S, Giles F, Talpaz M, Cortes J.** Dasatinib (BMS-354825) is active in Philadelphia chromosome-positive chronic myelogenous leukemia after imatinib and nilotinib (AMN107) therapy failure. *Blood.* 2007; 109: 497–9.
62. **Elrick LJ, Jorgensen HG, Mountford JC, Holyoake TL.** Punish the parent not the progeny. *Blood.* 2005; 105: 1862–6.
63. **Pfeifer H, Wassmann B, Pavlova A, Wunderle L, Oldenburg J, Binckebanck A, Lange T, Hochhaus A, Wystub S, Bruck P, Hoelzer D, Ottmann OG.** Kinase domain mutations of BCR-ABL frequently precede imatinib-based therapy and give rise to relapse in patients with de novo Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL). *Blood.* 2007; 110: 727–34.
64. **Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, Graham JA, Demur C, Hercend T, Diu-Hercend A, Su M, Golec JM, Miller KM.** VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth *in vivo. Nat Med.* 2004; 10: 262–7.
65. **Doggrell SA.** Dawn of Aurora kinase inhibitors as anticancer drugs. *Expert Opin Investig Drugs.* 2004; 13: 1199–201.
66. **Carter TA, Wodicka LM, Shah NP, Velasco AM, Fabian MA, Treiber DK, Milanov ZV, Atteridge CE, Biggs WH, 3rd, Edeen PT, Floyd M, Ford JM, Grotzfeld RM, Herrgard S, Insko DE, Mehta SA, Patel HK, Pao W, Sawyers CL, Varmus H, Zarrinkar PP, Lockhart DJ.** Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. *Proc Natl Acad Sci USA.* 2005; 102: 11011–6.
67. **Young MA, Shah NP, Chao LH, Seeliger M, Milanov ZV, Biggs WH, 3rd, Treiber DK, Patel HK, Zarrinkar PP, Lockhart DJ, Sawyers CL, Kuriyan J.** Structure of the kinase domain of an imatinib-resistant Abl mutant in complex with the Aurora kinase inhibitor VX-680. *Cancer Res.* 2006; 66: 1007–14.
68. **Giles FJ, Cortes J, Jones D, Bergstrom D, Kantarjian H, Freedman SJ.** MK-0457, a novel kinase inhibitor, is active in patients with chronic myeloid leukemia or acute lymphocytic leukemia with the T315I BCR-ABL mutation. *Blood.* 2007; 109: 500–2.
69. **Cheetham GM, Charlton PA, Golec JM, Pollard JR.** Structural basis for potent inhibition of the Aurora kinases and a T315I multi-drug resistant mutant form of Abl kinase by VX-680. *Cancer Lett.* 2007; 251: 323–9.